A SEX-SPECIFIC MOLECULAR AND BEHAVIOURAL ANALYSIS OF THE IMPACTS OF OBESOGENIC DIET EXPOSURE AND PROBIOTIC TREATMENT: INSIGHTS INTO SPECIFIC NUTRITIONAL DETERMINANTS OF HEALTH AND DISEASE

by

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Dalhousie University is located in Mi'kma'ki, the ancestral and unceded territory of the Mi'kmaq. We are all Treaty People.

DEDICATION PAGE

THIS DISSERTATION IS DEDICATED TO MY LATE GRANDMOTHER, WENDY, WHO PASSED AWAY BEFORE SEEING ITS COMPLETION.

♡

I will be forever grateful for her relentless support, love, and optimism.

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ABSTRACT

Nutrition-related environmental exposures (e.g., diet ingredients, vitamins, probiotic strains) have been reported to impact the composition of the gut microbiota, the microbiota-associated metabolites produced, and host health. This microbiota-gut-brain axis is related to a variety of behavioural and physiological outcomes, including anxiety or anxiety-related behaviours, feeding and obesity development, along with metabolic and immune-related hormonal responses. As well, the relationship between anxiety and obesity, with respect to how each affects the development and severity of the other, remains to be fully explained. Thus, the work in this dissertation focused on behavioural and physiological responses of male and female Long–Evans rats exposed to different types of purified and non-purified rodent diets, in isolation or in combination with the administration of the commercially available probiotic formulation, CEREBIOME®. The first study examined differences in the health-related effects of a commonly used Western diet and two of its control diet formulations (i.e., a high-carbohydrate protein-matched purified control compared to a commonly administered standard laboratory rodent chow). Next, these diet-specific alterations were expanded by including the additional factor of probiotic treatment to study the potential mitigative effects of a combination of two bacterial strains on poor diet exposure. The results of both studies highlight the differential health-related outcomes that result from the administration of specific experimental or control diets, along with specific strains of probiotic bacteria. Indeed, the work herein and emerging studies in this area are consistently highlighting diet-specific and probiotic strain-specific health outcomes. As emphasized more in the thesis, male and female rodents do not respond to these experimental manipulations in the same ways. Thus, characterizing sex differences in response to environmental factors that impact disease risk or severity is one vital component of studying the development and prevention of health and disease. Studies such as the ones presented in this dissertation advance our understanding of how nutritional factors contribute to psychological and physiological health outcomes in the hopes that we can work to improve prevention and treatment options for these accompanying disease states.

LIST OF ABBREVIATIONS USED

UNITS

%	Percentage
° C	Degrees Centigrade
m	Meter
cm	Centimeter (10^{-2} m)
mm	Millimeter (10^{-3} m)
M	Molar
mM	Millimolar (10^{-3} M)
µM	Micromolar (10^{-6} M)
g	Gram
kg	Kilogram (10^{3} g)
mg	Milligram (10^{-3} g)
µg	Microgram (10^{-6} g)
ng	Nanogram (10^{-9} g)
pg	Picogram (10^{-12} g)
fg	Femtogram (10^{-15} g)
kcal	Kilocalorie
L	Liter
mL	Millilitre (10^{-3} L)
µL	Microlitre (10^{-6} L)
CFU	Colony-Forming Unit
IU	International Unit
U	Units
g	Standard Gravity
s	Second
m/s	Meters per Second
min	Minute
h	Hour
W	Watts
w/v	Weight by Volume
Gx	Gestational Day x
Px	Postnatal Day x

INFLAMMATORY/METABOLIC

BMI	Body Mass Index
DPP-IV	Dipeptidyl Peptidase-4
G-CSF	Granulocyte Colony-Stimulating Factor
GLP-1	Glucagon-Like Peptide-1
GM-CSF	Granulocyte/Macrophage-CSF

NEUROSCIENCE

ACTH	Adrenocorticotropic hormone
AgRP	Agouti-Related Peptide
ARC	Arcuate Nucleus
AVP	Arginine Vasopressin
BDNF	Brain-Derived Neurotrophic Factor
CA3	*Cornu Ammonis* Subfield 3
cAMP	Cyclic Adenosine Monophosphate
CORT	Corticosterone
CRF	Corticotrophin-Releasing Factor
EPM	Elevated Plus Maze
F	Female
GABA	Gamma-Aminobutyric Acid
GAS	General Adaptation Syndrome
GR	Glucocorticoid Receptor
HCD	High-Carbohydrate Diet
HFD	High-Fat Diet
HPA	Hypothalamic-Pituitary-Adrenal
LDB	Light-Dark Box
M	Male
MC2-R	Melanocortin Type 2 Receptor
MD	Mediterranean Diet
MGB	Microbiota(biome)-Gut-Brain
(m)OFT	(modified) Open Field Test
MR	Mineralocorticoid Receptor
NSFT	Novelty-Suppressed Feeding Task
PL	Placebo
POMC	Proopiomelanocortin
PR	Probiotic
PVN	Paraventricular Nucleus
SD	Standard Diet
WD	Western Diet

MICROBIOLOGY/MOLECULAR BIOLOGY

bp	Base Pairs
BSA	Bovine Serum Albumin
Cq	Quantification cycle
ΔCq	Delta Cq
ΔΔCq	Double Delta Cq
CV	Coefficient of Variation
DMS	Dimethyl Sulfide

GRO/KC	Growth-Related Oncogene/Keratinocyte Chemoattractant
IFN-γ	Interferon gamma
IL-x	Interleukin-x
M-CSF	Macrophage-CSF
MCP-1	Monocyte Chemoattractant Protein-1
MIP-1α	Macrophage Inflammatory Protein-1 alpha
MIP-3α	MIP-3 alpha
PAI-1	Plasminogen Activator Inhibitor-1
RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted
T2DM	Type II Diabetes Mellitus
TNF-α	Tumour Necrosis Factor Alpha
VEGF	Vascular Endothelial Growth Factor
DNA	Deoxyribonucleic Acid
cDNA	Complementary DNA
ELISA	Enzyme-Linked Immunosorbent Assay
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
LPS	Lipopolysaccharide
NTC	No Template Control
NPY	Neuropeptide Y
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl Fluoride
qPCR	Quantitative Polymerase Chain Reaction
R^2	Coefficient of Correlation for the Standard Curve
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
mRNA	messenger RNA
RPL13a	Rat Ribosomal Protein L13a
RT-qPCR	Reverse Transcription qPCR

IMMUNOLOGY

APC	Antigen-Presenting Cell
MHC	Major Histocompatibility Complex
TCR	T-Cell Receptor
Th	Helper T cell

TAXONOMY

A.	*Akkermansia* (Genus)
B.	*Bifidobacterium* (Genus)
E.	*Escherichia* (Genus)
L.	*Lactobacillus* (Genus)

STATISTICAL TERMS

α	Alpha
η^2	Eta Squared
ANCOVA	Analysis of (Co)Variance
BORIS	Behavioral Observation Research Interactive Software
F-value	ANOVA Statistic (Fisher)
M	Mean
N	Total Sample Size
n	Partial Sample Size
ns	Not Significant
p-value	Probability Value
r	Correlation Coefficient
SD	Standard Deviation

OTHER

16S	Component of 30S (Small) Ribosomal Subunit (Prokaryotic)
60S	Large Ribosomal Subunit (Eukaryotic)
cat. #	Catalogue/category number
DIN	Drug Identification Number
DOHaD	Developmental Origins of Health and Disease
GAD	Generalized Anxiety Disorder
NaCl	Sodium Chloride
PTSD	Post-Traumatic Stress Disorder
SCFA	Short-Chain Fatty Acid
SNP	Single Nucleotide Polymorphism

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CHAPTER 1 INTRODUCTION

1.1 NUTRITIONAL IMPACTS ON HEALTH AND DISEASE

1.1.1 Nutrition and the Prenatal Environment

From the moment of conception, and even before, the external environment begins playing a role in the risk of health and disease across the lifespan. This idea of early life environment contributing to adult health outcomes was proposed by Barker and Osmond (1986) when they postulated a link between poor early life nutrition and increased risk of coronary heart disease mortality later in adulthood. The concrete consideration that early life diet after birth might affect, or 'program', long-term health consequences has been around for more than 30 years (Lucas, 1991). However, even before that time, rodent studies linked undernutrition to developmental health outcomes (e.g., weight gain in Widdowson & McCance, 1963; organ development in Jackson, 1932). The first mention of the Fetal Origins of Adult Disease hypothesis was in a study on associations of low birthweight combined with large placentas and increased risk of hypertension in adulthood (Barker et al., 1990). The idea of developmental plasticity has expanded over the past three decades, with many implications for health and disease risk. For example, poor quality maternal nutrition and exposure to pollutants have been linked to offspring development of conditions such as obesity and asthma (reviewed by Aris et al., 2018). Broadly, when talking about prenatal environmental exposures, high levels of glucocorticoid exposure to the fetus, interference of hypothalamic-pituitary-adrenal (HPA) axis development (e.g., from maternal stress, infection), problems with organ development, and epigenetic alterations that change gene expression are the major mechanisms that can affect later health and disease risk (summarized by Mandy & Nyirenda, 2018).

Aside from maternal undernutrition and later risk of metabolic dysfunction, uteroplacental blood flow, placental function, and fetal metabolism have all been linked to postnatal health outcomes (early review by Gillman, 2005). As such, the Fetal Origins of Adult Disease hypothesis has since expanded to the Developmental Origins of Health and Disease (DOHaD) hypothesis (i.e., incorporating the importance of both the prenatal and postnatal

developmental periods; Gillman, 2005). Undeniably, the DOHaD hypothesis has been described as "one of the most important theories in biological science" (Suzuki, 2018, p. 266). As described by Suzuki (2018), the concept of DOHaD can be considered an extension of the gene–environment interaction theory (i.e., that both genes and the environment contribute to development; see Anastasi, 1958; Plomin et al., 1977; Scarr & McCartney, 1983) because the DOHaD hypothesis delves into the mechanisms for why certain genes, in certain environments, yield developmental phenotypic variation. For instance, Hales and Barker (1992) proposed the 'thrifty' phenotype hypothesis to explain the etiology of type 2 diabetes mellitus (T2DM). In the thrifty phenotype hypothesis, it was postulated that inadequate early nutrition (fetal and early postnatal) could lead the pancreas or other endocrine systems to learn to conserve energy stores (Hales & Barker, 1992). Specifically, it is proposed that these systems become thrifty with the energy they are given because they are accustomed to insufficient available energy (e.g., impairing growth of Beta cells and the islets of Langerhans), which contributes to the later development of T2DM (Hales & Barker, 1992). Important to this hypothesis is if there is a 'mismatch' in nutritional state postnatally from what the individual experienced prenatally, then this would put them at higher risk of various non-communicable diseases (Cleal et al., 2007; Gluckman et al., 2008).

1.1.2 Nutrition and the Postnatal Environment

Postnatal environmental exposures can also affect the risk of health and disease, especially in the critical period of development from conception until two years of age (Barker et al., 2008). Importantly, these environmental and nutritional impacts are not acting in isolation; there are also genetic predispositions to diseases that are impacted by the environment in which they exist (Langley-Evans, 2015), in part due to epigenetic responses to the environment (Jaenisch & Bird, 2003; Waterland & Jirtle, 2003). For example, presence of the rs9939609 allele in the *FTO* gene (i.e., a single nucleotide polymorphism [SNP], thymine to adenine, see Frayling et al., 2007) is associated with increased body mass index (BMI) in young adulthood (Sovio et al., 2011), but longer exclusive breastfeeding can be protective in both males and females in developing that 'increased BMI' phenotype as

measured in adolescence, even if the aforementioned allele is present (Abarin et al., 2012). Broadly, in infancy, infections, antibiotics, and vaccines are all environmental exposures that have been shown to impact the risk of disease later in life (reviewed by Raymond et al., 2017). For instance, in one study, both prenatal low gestational weight gain (in female infants) and postnatal septicemia in preterm neonates reduced the risk of developing asthma in childhood (Grischkan et al., 2004).

Importantly, the development of health and disease outcomes can be influenced by risk factors outside the prenatal and early postnatal period, with impacts during pre-pregnancy (to mothers and fathers; reviewed in Hieronimus & Ensenauer, 2021) and early childhood also being shown to impact offspring health (e.g., Normia et al., 2013). In this work by Normia and colleagues (2013), it was reported that maternal carbohydrate (i.e., high) and fat intake (i.e., high and low, not mid-range) during pregnancy were predictors of childhood systolic blood pressure at four years old, but childhood weight and fat intake at four years old were also significant predictors of systolic blood pressure. Furthermore, while no significant maternal predictor variables were found for childhood diastolic blood pressure, childhood weight at four years old was significantly associated with diastolic blood pressure (Normia et al., 2013). Another example of a risk factor for disease well into the childhood period would be the research on childhood bullying and later disease promotion in adulthood. For instance, victims of childhood bullying have been reported to have increased systemic inflammation (Copeland et al., 2014) and increased BMI and waist-to-hip ratio (Baldwin et al., 2016) in adulthood. As such, any discussion of factors that contribute to the development of disease or that promote health should include social determinants of health (e.g., education, social support, poverty). In fact, one review estimated that deaths in the United States linked to social influences are just as prevalent as deaths linked to behavioural or physiological causes (e.g., smoking, diet; Galea et al., 2011). As stressed by Braveman and Gottlieb (2014), these determinants of health are heavily interrelated (e.g., less access to healthcare or healthy foods can worsen genetic or environmental risk of disease development) and should not be studied in isolation.

Taken altogether, the research presented in Sections 1.1.1 and 1.1.2 demonstrates key concepts that are relevant to this thesis: 1) both prenatal and postnatal influences affect

offspring health outcomes; 2) environmental and nutritional impacts can continue to affect health outcomes after the traditional 'critical period' of 0 to 2 years of age; and 3) environmental-related programming impacts can interact with genetic and epigenetic factors in the preconception period, prenatal period, postnatal period, and throughout life. For instance, as reviewed by Gensous et al. (2019), animal studies are showing that nutritional interventions, like calorie restriction, seem to impact epigenetic mechanisms related to aging (e.g., by reducing epigenetic drift that would contribute to aging, see Maegawa et al., 2017). Furthermore, although much of the previous work that has been discussed has focused on prenatal and postnatal factors affecting the development of later metabolic dysfunction and other physical diseases, important to this discussion is that mental health and mental illness are key targets for DOHaD research. In 2010, Insel and Wang challenged researchers to reconsider how mental illness is categorized and conceptualized because research establishing the importance of genetic risk and the environmental context in mental illness development was paralleling that of physical illnesses. More recently, the DOHaD hypothesis has been a central consideration when studying the onset and prevention of mental health disorders. For instance, it is well-established that maternal factors during pregnancy (e.g., diet, exercise) seem to be key for the later development of neurodevelopmental and psychological disorders (Van Lieshout & Krzeczkowski, 2016).

1.1.3 Introduction to the Gut Microbiota

To summarize, nutritional effects long before and long after birth impact both physiological and psychological health outcomes. While nutrition and other environmental impacts interact with genetics, in part via epigenetic modifications, the microbiota also plays a major role in the relationship between genetics, the environment, and heath (Stiemsma & Michels, 2018). The human microbiota is often thought of as synonymous with the human gut microbiota as an overwhelming number of studies focus on this major interface of host-microbial symbiosis in the gut (i.e., with bacteria, fungi, protists, viruses, archaea). To name a few, studies of vaginal (e.g., White et al., 2011), lung (e.g., Shukla et al., 2017), and oral (e.g., Krishnan et al., 2017) microbiota are unravelling clinically relevant

mechanisms related to health and disease risk in humans and animals. For instance, bacterial infection in the uterus during pregnancy can result in preterm birth, which has been linked to an associated inflammatory response in this region (Jefferson, 2012). The presence of specific inflammatory markers in cervicovaginal fluid has also been associated with preterm labour and rupture of membranes (Park et al., 2020).

As just described, other organs and systems harbour microbiota that are crucial for host success but the gut microbiota (e.g., stomach, small intestine, large intestine, feces) and its interactions with health and disease outcomes will be the focus of this dissertation. Bacteria and other microbes in the gut help synthesize nutrients, digest food, regulate immune activity, and contribute to nervous system functioning (Y.-J. Zhang et al., 2015). The human gut microbiota is estimated to comprise nearly 200 individual strains of bacteria alone (Faith et al., 2013). Furthermore, the collective set of genes of the gut microbiota (i.e., the gut microbiome; 99% bacterial) has been reported to be about 150 times larger than the human genome (Qin et al., 2010). In contrast, when looking at cell numbers rather than gene numbers, a recent estimate by Sender et al. (2016) reports that the number of bacteria in the body (i.e., bacterial cells) is likely approximately equal to that of human cells (i.e., both in the order of 10^{13}).

In human infants, the gut microbiota composition has been estimated to begin resembling that of an adult (e.g., similar genera, more temporal stability) at around one year of age (C. Palmer et al., 2007). As well, the diversity of the gut microbiota (variation and number of taxa) increases after birth and peaks in early adulthood Belizário and Napolitano (2015), with greater individual variability and declines in diversity reported in old age (Jeffery et al., 2012). The bidirectional communication between the microbes in the gut and the brain has been termed the microbiota-gut-brain (MGB) axis (Cryan et al., 2019; Cryan & O'Mahony, 2011; Grenham et al., 2011) or the brain-gut-enteric-microbiota axis (Rhee et al., 2009). This communication between the body and the commensal microbiota is regulated and affected by a variety of bodily systems (e.g., neural, endocrine, immune; Grenham et al., 2011). Interestingly, when the fecal gut microbiota of fraternal and identical twins is compared, both types of twins are more similar in their gut microbiota to each other than to their mothers, but there is no difference between the degree of similarity

by type of twin pairs (i.e., identical twins were not more similar than fraternal; Turnbaugh, Hamady, et al., 2009), demonstrating a clear role for both genetics and the environment (e.g., *in utero*, during birth, postnatal) in shaping the development of the gut microbiota.

In the human gut, anaerobic bacteria are much more abundant than aerobic bacteria, and more specifically, the Bacteroidetes and Firmicutes phyla together can comprise up to 90% of total bacteria (Neish, 2009). The human gut microbiota has also been reported to have a lower abundance of species from the Proteobacteria, Actinobacteria, Verrucomicrobia, and Fusobacteria phyla (Eckburg et al., 2005). In F344 rats (male and female), the Bacteroidetes and Firmicutes phyla are also highly abundant, along with lower abundances of Proteobacteria, Actinobacteria, and Verrucomicrobia (S. M. Lee, Kim, Park, et al., 2018; S. M. Lee, Kim, Yoon, et al., 2018). One comparative analysis of microbiota patterns in fecal samples from adult mice (male C57BL/6 mice), rats (male NIH heterogeneous stock rats), monkeys (female cynomolgus macaques), and humans (male and female) reported that, while all samples had a high abundance of Bacteroidetes and Firmicutes, mice and rats had more Bacteroidetes than Firmicutes, whereas monkeys and humans had more Firmicutes than Bacteroidetes (Nagpal et al., 2018). Actinobacteria were quite common in humans and nearly undetectable in mice, rats, and monkeys, whereas Proteobacteria were highest in rats, and Verrucomicrobia abundance did not vary significantly between subject types (Nagpal et al., 2018).

When discussing humans and animals with a central nervous system, the gut microbiota can interact bidirectionally with this nervous system through various means (Morais et al., 2021). As reviewed by Morais et al. (2021), the major established routes of communication between the central nervous system and the gut microbiota are the autonomic nervous system (e.g., vagus nerve), neuroendocrine system (e.g., hormone release from various organs), the HPA axis (e.g., by affecting cortisol release from the adrenal glands), the immune system, and metabolic pathways. Important to conceptualizing this communication is that the gut microbiota can produce various compounds and metabolites (e.g., neurotransmitters) and impact structural components of the gut (e.g., gut lining integrity) that can affect the function of various biological systems (e.g., hormone production, stress responding, immune responding; Morais et al., 2021; Rieder et al., 2017).

1.1.4 Dysbiosis

Although a full characterization of healthy or harmful microbial presence in humans and other animals is still being actively elucidated, people in good health have been reported to have species of *Lactobacillus*, Enterobacteriaceae, and *Enterococcus* in their small intestines, along with species of *Bacteroides* and Clostridia in their large intestines and end of the small intestine (Fiebiger et al., 2016). Arumugam and colleagues (2011) describe three clusters (enterotypes) of gut microbiota in humans based on a prevalent genus in each (i.e., *Bacteroides, Prevotella, Ruminococcus*) that are associated with the presence of other genera that can affect health. The authors hypothesized that characterizing individuals by enterotype could be a future marker for disease diagnosis and treatment plans (Arumugam et al., 2011). Even so, the concept of distinct enterotypes has been met with criticism (e.g., Jeffery et al., 2012) because these enterotypes seem to be highly dependent on environmental factors (e.g., diet; G. D. Wu et al., 2011), and they are an oversimplified explanation of the dynamic nature of the gut microbiota. Interestingly though, it does seem that at least for the more common and more studied *Bacteroides*- and *Prevotella*-dominated enterotypes, knowing which enterotype is present in an individual can aid with weight loss management strategies (e.g., high-fibre diets for people with the Prevotella enterotype, increasing members of *Bifidobacterium* (*B.*) for the Bacteroides enterotype; reviewed by Christensen et al., 2018).

If there is a disruption in the composition of gut microbes or their ability to communicate with the overall MGB axis, health problems might result depending on a variety of host characteristics (e.g., microbial changes can alter gut motility and result in functional or inflammatory gut pathology; Rhee et al., 2009). Both the composition of the gut microbiota and associated metabolites that are produced have been linked to increased metabolic disease risk (e.g., obesity, diabetes; reviewed by M. Sharma et al., 2020) and mental illnesses (e.g., anxiety, depression; reviewed by Clapp et al., 2017). For example, increased presence of bacteria from the class Clostridia has been associated with the development of autism, Alzheimer's disease, and T2DM, whereas reduced abundance of the genus *Lactobacillus* (*L.*) has been linked to both autism and T2DM (reviewed by Ghaisas et al., 2016).

More specifically, when the gut microbiota composition varies from what is optimal or favourable (i.e., from a eubiotic state) for an individual host, dysbiosis is said to occur (García-Montero et al., 2021; Lynch & Pedersen, 2016). As previously introduced, specific gut microbiota compositions have been linked to harmful host states, such as neurological disorders (e.g., Parkinson's disease, Alzheimer's disease, autism spectrum disorder; Suganya & Koo, 2020), psychological disorders (e.g., major depressive disorder; Suganya & Koo, 2020), metabolic dysfunction (e.g., obesity in Trasande et al., 2013; T2DM in Arora et al., 2021), and immune-related disease development (e.g., inflammatory bowel disease in Saez-Lara et al., 2015). For instance, one human study examined microbial patterns in adult subjects with or without overweight and obesity and found decreases in beneficial bacterial genera such as *Bifidobacterium* and increases in pathogens (e.g., *Fusobacterium*, *Escherichia* [*E.*], *Shigella*) in participants classified as 'overweight' or 'obese' (R. Gao et al., 2018). While the predominant population of gut microbes has been shown to be resistant to change (e.g., compositional changes from antibiotics or stress return to baseline in most people; Rhee et al., 2009), the gut microbiota is also described as being readily changeable in response to different host environments (García-Montero et al., 2021). Overall, as reviewed by Lynch and Pedersen (2016), dysbiosis has been associated with disease states in various body systems (e.g., neurobehavioural disorders, autism, cardiovascular disorders, atherosclerosis, respiratory disorders, asthma). However, optimal individual gut microbiota compositions are highly variable and still being explored.

One difficulty in conceptualizing dysbiosis and its effects on health is that the specific mechanisms for why dysbiosis affects the MGB axis more severely in some people compared to others are still being characterized. Gnotobiotic animal models, or, more specifically, germ-free models (i.e., usually rodent; no exposure to microorganisms and do not have a microbiota), can yield some insight into the behavioural and physiological consequences of a lack of a microbiota (Cryan et al., 2019). As well, colonization of germ-free mice with specific types of microbiota (e.g., from a conventional animal, from an animal with a disease phenotype, with specific strains of bacteria) can help to characterize the effects of those microbes in a physiological system (Martín et al., 2016). For instance, one pivotal study by Gareau and colleagues (2011) infected female specific pathogen-free

C57BL/6 and Swiss Webster mice (specific pathogen-free and germ-free) with *Citrobacter rodentium*, with or without additional stress (i.e., water avoidance) and measured the consequent memory function. After the infection with or without added stress, they additionally administered a probiotic (6×10^9 colony-forming units [CFU]/day of Lacidofil®; 95% *Lacticaseibacillus rhamnosus* R0011, 5% *L. helveticus* R0052; or placebo; Gareau et al., 2011). First, they found that in the conventional C57BL/6 mice, infection only impaired working hippocampal-dependent memory function (i.e., T-maze and novel object recognition test) when combined with stress, and memory function was normalized with probiotic treatment (Gareau et al., 2011). However, compared to the specific pathogen-free Swiss Webster mice, the germ-free Swiss Webster mice had severely impaired memory and no observed anxiety-related behaviours during testing at baseline, regardless of stress or infection (Gareau et al., 2011). These gnotobiotic studies can begin to provide clarity on the interactions of specific bacteria in the gut and the health-related consequences of dysbiosis, but a full understanding of what constitutes dysbiosis is dependent on research gaining more clarity on what exactly constitutes a healthy or unhealthy microbiota (Martín et al., 2016).

Although antibiotics have undoubtedly changed modern medicine, with Alexander Fleming (and E.B. Chain and H. W. Florey) being awarded the Nobel Prize in Physiology and Medicine in 1945 for the discovery of penicillin in 1928 (Alharbi et al., 2014), they have major impacts on the gut microbiota and lead to dysbiosis (usually temporary; e.g., doxycycline use is related to lower *Bifidobacterium* diversity; Elvers et al., 2020). In this respect, studying the effects of antibiotic treatment on the composition of the gut microbiota can be a valuable tool for elucidating MGB axis interactions because antibiotics temporarily deplete specific microbial populations (Cryan et al., 2019). In most individuals, gut bacterial diversity and abundance return to baseline within a few weeks (up to six months), but this finding has implications related to both the repeated use of antibiotics and the misuse of antibiotics (e.g., when not needed; Elvers et al., 2020). In one rodent study, antibiotic treatment with succinylsulfathiazole to mothers during the conception period (i.e., one month before breeding until gestational day 15) was shown to reduce social and exploration behaviours in male and female Wistar rat offspring in the post-weaning period (Degroote et al., 2016). With humans, antibiotic use is highly prevalent in children (e.g.,

amoxicillin was the most frequently prescribed pediatric medication in the US in 2010; Chai et al., 2012), and antibiotic use has been linked to different types of dysbiosis (Vangay et al., 2015). Interestingly, one study, with nearly 30,000 mother-child pairs, reported that antibiotic use in early life (before six months of age) was related to increased risk of being 'overweight' in childhood (seven years old) when mothers were of 'normal' weight but decreased risk of being 'overweight' when mothers had elevated pre-pregnancy BMI (i.e., greater than 25 kg/m^2; Ajslev et al., 2011).

1.1.5 Healthy and Unhealthy Diets

The literature has consistently demonstrated that diet components and quality can affect mental and physical health. It should be noted that research on what constitutes a healthy diet is always evolving and highly individualized (e.g., a low-carbohydrate diet is beneficial for treating some cases of T2DM or obesity but should not be generalized to the entire population; Katz & Meller, 2014). It is presently most widely accepted that what constitutes a healthy diet is a pattern of eating that provides sufficient macro and micronutrients, comprises an appropriate number of calories for bodily function, and gives access to ingredients that have been documented to be associated with health benefits, while limiting ingredients that are harmful to health (Katz & Meller, 2014; Kopp, 2019). Perhaps the most known 'healthy' dietary pattern, the Mediterranean diet (MD) and its variations, are often studied for their health benefits (Cena & Calder, 2020). As reviewed by Katz and Meller (2014), the MD has been heavily researched and reported to be related to a longer life span, cognitive benefits, and a lower risk of cancer and cardiovascular health issues. The MD emphasizes plant-based and fibre-rich foods (e.g., seeds, legumes, cereals, olive oil) along with specific types of meats (e.g., seafood is preferred over red meat; Bach-Faig et al., 2011). As such, the MD is a contrasting dietary pattern to the Western diet (WD; i.e., a diet rich in processed, refined, and high-sugar foods; Jacka et al., 2010). Broadly, WDs can result in increased insulin production after feeding, which can dysregulate the body's response to insulin and result in metabolic consequences, such as increased fat storage, appetite, and weight gain (Kopp, 2019).

Diet-induced obesity rodent models are one way to study the progression of obesity or the

response to factors that affect obesity development or its treatment. Of course, genetics plays a role in the etiology of obesity and poor metabolic health, but diet has been described as one of the most important environmental risk and protective factors for obesity development and associated consequences (Bastías-Pérez et al., 2020). In rodents, high-fat diets (HFDs) have been broadly described as diets with at least 30% of their energy derived from fat sources (commonly up to 60% or greater; Buettner et al., 2007). With knowledge gained from human studies and observations showing negative health consequences of WD consumption, rodent WDs that are both high in unhealthy fat (e.g., saturated fat) and carbohydrate sources (e.g., sucrose) are used to model a version of human WD consumption (Hintze et al., 2018). Cafeteria-style diets are less commonly used but usually consist of standard, or normal laboratory chow, diets (SDs) with added WD-style human foods (e.g., sweet foods, salty foods, processed foods; Lalanza & Snoeren, 2021). As reviewed by Buettner et al. (2007), the type of rodent strain (e.g., Wistar rats, Long–Evans rats, C57BL/6 strains of mice), source of fat (e.g., lard, milk fat, safflower oil), amount of fat (e.g., 25% kcal fat, 45% kcal fat, 60% kcal fat), and diet administration length (e.g., 21 days, 70 days, 300 days) can be highly variable study-to-study. Nonetheless, diet-induced obesity rodent models (e.g., HFD-induced obesity, WD-induced obesity, cafeteria diet-induced obesity) have the goal of complementing genetic models of obesity (e.g., leptin-deficient *ob*/*ob* mice, leptin receptor-deficient *db*/*db* mice or Zucker rats, transgenic corticotrophin-releasing factor[CRF]-overexpressing mice) and pharmacologically or surgically induced obesity models (e.g., hippocampal lesions, ovariectomy leading to estrogen deficiency, streptozotocin; reviewed by Islam & Wilson, 2012; Vangoori et al., 2022).

Terminology for experimental HFDs, WDs, and cafeteria diets is often inconsistent between studies (e.g., the same purified diet can be called an HFD or WD, depending on the study). Although inconsistencies can often be verified by reading the methodology in the studies, diet product numbers and compositions are not always reported or reported in different ways, making comparisons difficult if the diet product numbers are unavailable. As such, it should be clarified that for the purpose of this literature review and dissertation, the term ‘HFD’ will be used to describe a diet that derives 60% or greater of its energy from fat (a common cut-off described by Buettner et al. (2007), whereas the term ‘WD’

will be used for diets that are designed with increased fat that approximates the increased carbohydrates (e.g., 40% kcal fat, 40% kcal carbohydrate) and of which the sources of fat and carbohydrates have been demonstrated to negatively affect health (e.g., lard, butter, sucrose). Finally, although the drawbacks and benefits of using cafeteria diets have been debated elsewhere (Bortolin et al., 2018; Murphy & Mercer, 2013; Sampey et al., 2011), when literature using cafeteria diets is being presented herein (e.g., González et al., 2023; Lang et al., 2019; Maniam & Morris, 2010), attempts will be made to be as detailed as possible when describing the diets.

Paralleling the inconsistencies in experimental diet terminology, the use and descriptions of control diets for the experimental diets can be highly variable between studies (e.g., the term 'control diet' can refer to an SD or purified nutrient-matched diet). Regarding the various ways to control for experimental diet manipulations with control diets, the term 'SD' will be used to refer to cereal-based chow diets that are not designed to match the experimental diet but are commonly given to rodents in research capacities regardless of a diet manipulation (i.e., deemed 'SDs' regardless of the macronutrient breakdown). The term 'HCD' (high-carbohydrate diet) will refer to purified control diets that are designed to be nutrient matched to the experimental diets but at the expense of reduced fibre content and increased carbohydrates. However, it should be noted that even though these HCDs are high-carbohydrate diets, the source of the carbohydrate is often altered to a less metabolically harmful source (e.g., see B. Wang et al., 2009 for a comparison between sucrose-based and corn-starch based diets). For instance, an HCD used as a control for a WD is often corn starch- or maltodextrin-based instead of sucrose-based like the WD. In saying this, as recently reviewed by Jung and Choi (2017), defining HCDs is not standardized, despite these diets being shown to impair metabolic health, but the authors suggest that any diet with 55% or greater of its energy derived from carbohydrate sources to be considered an HCD. Finally, in this dissertation, special cases where purified nutrient-matched control diets have been altered in some way will be explicitly stated as such (e.g., to reduce carbohydrates or increase fibre; as in Lang et al., 2019; Sasidharan et al., 2013). Relatedly, the general term 'low-fat diet' will be avoided when discussing rodent studies as this term is often used interchangeably with terms such as 'standard diet, 'control diet',

and ‘high-carbohydrate diet’, and, as such, the terms SD or HCD (as per the definitions above) will be preferred.

1.1.6 Diet and the Gut Microbiota

Along with factors such as pollution, stress exposure, and various host characteristics (e.g., sex, presence of illness), it has been reported that diet is one of the most, if not the most, important environmental factors that shape the gut microbiota (Gacesa et al., 2022; Y. S. Kim et al., 2020; Voreades et al., 2014). Previous work has found that, compared to SD, rodents given an HFD (60% kcal fat, 20% kcal carbohydrate) had increased levels of specific taxa from the Firmicutes phylum, such as genera *Lactococcus* and *Dorea* in caecal contents (i.e., six weeks to male Sprague–Dawley rats starting in adolescence; Crawford et al., 2019), along with increased *Coprococcus* and *Ruminococcus* in fecal samples (i.e., 80 weeks to adult male C57BL/6 mice; Velázquez et al., 2019). Interestingly, an often overlooked diet component, fibre, has been shown to have more of an impact on the gut microbiota than diets high in carbohydrates or fats. Namely, adult male and female C57BL/6:129 mice that transitioned from an SD that contained soluble dietary fibre to either a refined WD or control HCD had distinct changes (e.g., taxonomic shifts such as a decrease in the family Prevotellaceae) in their microbiota compared to when they were fed the SD (Morrison et al., 2020).

In humans, diet choice based on cultural- and location-specific factors can impact which microbes are found in the gut. One species of bacteria, *Bacteroides plebeius* M12, isolated from the feces of Japanese men and women (Kitahara et al., 2005), was later linked to aiding with dietary seaweed digestion through the production of specific bacterial metabolites (i.e., porphyranases and agarases; Hehemann et al., 2010). Indeed, the components of diets have far-reaching effects on the gut microbial community, and even short-term dietary changes can yield taxa shifts. For instance, David et al. (2014) administered either an animal-based (e.g., eggs, meats, cheese) or a plant-based diet (e.g., vegetables, rice, lentils) to humans for five days and measured gut microbial presence in feces. Subjects in the animal-based diet group had increases in genera of bacteria known to be bile-tolerant (e.g., *Bacteroides*) and reductions in Firmicutes that break down dietary

plant fibre (e.g., *Roseburia*), whereas fewer differences in microbe presence from baseline were found with the plant-based diet (David et al., 2014). In another study, G. D. Wu et al. (2011) examined the effects of high-fat/low-fibre or low-fat/high-fibre diet administration for ten days and noted changes in the microbiota compositions even after one day. However, they also found that the two enterotypes (see Section 1.1.4) distinguished in participants (i.e., *Bacteroides*-dominated in participants that consumed animal protein and *Prevotella*-dominated in those that consumed more carbohydrate and plant-based foods) were stable with short-term diet manipulation (G. D. Wu et al., 2011).

1.1.7 Probiotics and the Gut Microbiota

Probiotics, as they are thought of today, gained scientific interest in the early 20th century, when Élie Metchnikoff, a Ukrainian zoologist and immunologist, observed that eating fermented dairy products was related to better health (e.g., digestion) and longevity in Bulgarian people (Anukam & Reid, 2008; Metchnikoff, 1907). Of course, this idea was informed by the observations of Stamen Grigorov, who isolated species of bacteria in Bulgarian yogurt (Kisseli-mléko; Grigorov, 1905). Today, there is some ambiguity in the literature on classifying probiotics, but they are generally accepted as part of the 'functional foods' category (Damián et al., 2022; D. C. Lin, 2003). More officially, probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (C. Hill et al., 2014, p. 507). As outlined in detail recently by N. Sharma et al. (2023), probiotics can also be broken down into specific categories based on the purpose of their administration (e.g., nutribiotics are probiotics that generate beneficial nutritional components; psychobiotics are probiotics that specifically promote beneficial psychological outcomes; see also Chaudhari & Dwivedi, 2022; Dinan et al., 2013). Furthermore, other compounds that can affect health outcomes by interacting with consumed or already present microbes in a host are actively being characterized in research. For instance, prebiotics are compounds (e.g., specific food ingredients, fibre) that interact with microbes in a host to benefit a specific health outcome (Gibson et al., 2017) and, when combined with a probiotic, are called synbiotics (Swanson et al., 2020).

Within the category of probiotics that meet this aforementioned definition (i.e., specific strain(s) of live microbes at a specific dose for a specific health benefit) are probiotic supplements (e.g., capsules, powders) and probiotic foods (i.e., foods that contain probiotics; C. Hill et al., 2014; Homayoni Rad et al., 2016). It should be mentioned that, within the sub-category of probiotic foods, fermented foods can be deemed 'probiotic' (i.e., probiotic fermented foods) if these foods have specific dosages and strains of microbes (added or naturally occurring) and have been studied for a health benefit (J. Gao et al., 2021; Marco et al., 2021). A claim that a food 'contains probiotics' can also be made even if there is a lack of evidence on strain-specific benefits (Marco et al., 2021). Otherwise, even if a fermented food does not meet the definition of a probiotic, it can still be claimed to 'contain live and active cultures' if there would be live microbes present when it is consumed (e.g., kombucha, miso, yogurts that do not meet the definition of a probiotic; Marco et al., 2021). Other fermented foods that do not contain live microbes at the time of consumption (e.g., leavened breads, beer, fermented foods that have been heated or pasteurized) are not considered to be probiotics or contain live microbes (Marco et al., 2021). However, despite attempts to clearly define and study the benefits of specific probiotics, due to the lack of strict regulations, many food and supplement products deemed probiotics do not meet the definition of a probiotic (Reid, 2016).

The overarching premise of probiotics is that their use can, in some way, benefit health (e.g., digestive health, immune health, vaginal health) by helping to create a favourable environment for this benefit (C. Hill et al., 2014), although overarching mechanisms for strain-specific benefits are still scantily understood (Marco et al., 2006; Widyastuti et al., 2021). Indeed, the field of probiotics has exploded in the past few decades, with research on mechanisms of action of specific probiotic strains or combinations, along with clinical studies increasing each year (McFarland, 2015). Besides specific human and animal studies related to the application of probiotics for preventing or treating various symptom profiles or disease states, probiotics have also been gaining popularity for other applications. For instance, probiotics can be used in aquaculture (e.g., improving water quality, lowering risk of disease; Gatesoupe, 1999; Midhun et al., 2023), agriculture (e.g., reducing absorption of consumed pesticides, for soil fertilization, as a biopesticide; Anderson & Kim, 2018; de Souza Vandenberghe et al., 2017; Trinder et al., 2016), and food science

(e.g., improving food texture or quality, reducing food contaminants; Guimarães et al., 2020; Popova, 2017; Sadat Mirmahdi et al., 2021).

Most commercially available probiotics are comprised of strains of Gram-positive lactic acid bacteria, either on their own or in combination with other strains (Behnsen et al., 2013; Cross et al., 2004). However, less commonly discussed Gram-negative bacteria can also have probiotic properties (e.g., *E. coli* Nissle 1917 can be used to treat constipation and colitis; Nissle, 1959, as reviewed in Behnsen et al., 2013; *Akkermansia, A., muciniphila* improves metabolic disease and inflammatory bowel disease outcomes; reviewed in Rodrigues et al., 2022). Gram-positive bacteria that produce lactic acid (e.g., lactobacilli, bifidobacteria, streptococci) are almost exclusively non-pathogenic and are commonly used to develop probiotics because these bacteria function (and survive) in the gut by using the acids they produce (e.g., lactic, acetic) to prevent the growth and survival of harmful bacteria, along with synthesizing beneficial compounds (e.g., enzymes, vitamins) to aid with host digestion (Parvez et al., 2006; N. Sharma et al., 2023). As cautioned by Fijan (2014), probiotics are generally safe and well-tolerated by people in good health, but certain probiotics can be contraindicated in certain groups (e.g., *Saccharomyces boulardii* and *Bacillus subtilis* in immunocompromised individuals). Specific strains of lactobacilli and bifidobacteria are often characterized and developed for use as probiotics from dairy products and human guts (N. Sharma et al., 2023). For instance, lactobacilli probiotics are a common addition to antibiotic and antifungal treatment plans for vaginal infections, such as bacterial vaginitis (often from *Gardnerella vaginalis*) and vulvovaginal candidiasis (often from the yeast *Candida albican*s) because they seem to both help with treating the primary infection, but also help with the dysbiosis-associated symptoms of the primary treatment (J.-M. Kim & Park, 2017).

Importantly, many tested probiotics are transient and may exert their effects without colonizing the areas upon which they act. In one study with male and female CD-1 neonatal (8-day-old) mice, Preidis et al. (2012) administered two different *Limosilactobacillus reuteri* strains derived from humans or a placebo vehicle and tracked their transit through the mouse gastrointestinal system. Even though mice administered either probiotic strain had changes in cellular functioning (e.g., intestinal cell migration) and increased fecal

microbiota diversity after only one dose, *Limosilactobacillus reuteri* (a species that is not present in neonatal pups) was only detected in the distal ileum for a few hours after probiotic administration and was completely absent 24 hours after administration (Preidis et al., 2012). Of course, more research is needed on how probiotics travel through and persist in the gut of animals and humans, especially characterizing differences with specific strains, their combinations, and factoring in subject characteristics (e.g., rodents exposed to stress have changes in gut transit time; Tache & Perdue, 2004). A recent study in humans administered a probiotic (30×10^9 CFU per day; 45% *L. helveticus* R0052, 17% *Lacticaseibacillus paracasei* HA-108, 16% *B. breve* HA-129, 6% *B. longum* R0175, and 6% *Streptococcus thermophilus* HA-110) to characterize the passage and persistence of the strains in fecal samples (Tremblay et al., 2023). The authors show that four strains appeared in the fecal samples between one and two days after starting treatment, and three of these strains persisted for an average of less than a week (i.e., R0052, HA-108, and HA-129), whereas R0175 persisted for an average of 8.5 days and greater than 15 days in some participants (Tremblay et al., 2023). Interestingly, longer persistence (i.e., greater than 15 days) of R0175 was found with participants classified as having intermediate gut transit time, and certain genera that were present at baseline in these participants were associated with longer R0175 persistence (e.g., increased *Blautia*, reduced *Bifidobacterium*; Tremblay et al., 2023).

Even with this idea that probiotics can be used to impart a therapeutic health benefit, the mechanisms for specific strain benefits are still being actively elucidated by *in silico*, cellular, agricultural, animal, and human studies (e.g., Devika et al., 2021; Mazhar et al., 2023; Mazziotta et al., 2023; Miller et al., 2019; Rahman et al., 2018). An early study by Desbonnet et al. (2008) provided some insight into the possible role of one probiotic, *B. longum longum* 35624 (administered in drinking water to male Sprague–Dawley rats), in affecting MGB axis function. Specifically, treatment with this probiotic (compared to vehicle placebo) increased plasma levels of tryptophan (a precursor of serotonin), and its metabolite (kynurenic acid), along with increasing 5-hydroxyindoleacetic acid (a metabolite of serotonin) in the frontal cortex and 3,4-dihydroxyphenylacetic acid (a metabolite of dopamine) in the amygdaloid cortex (Desbonnet et al., 2008). Another study in humans by Guo et al. (2012) demonstrated that *Lacticaseibacillus casei* F0822 has

therapeutic potential for lowering serum cholesterol, in part because it can lower a compound called deoxycholic acid in the intestine because a surface-level protein on *Lacticaseibacillus casei* binds to a carboxyl group on deoxycholic acid. Overall, there is still considerable uncertainty as to how probiotics modulate health and disease, but the action of probiotics, at least in the gut, has been broken down into three levels: 1) at the gut lumen where these bacteria can interact with pathogenic microorganisms; 2) at the gut barrier where they can interact with the mucosal immune system, enteric nervous system, and digestive system; and 3) outside of the gut, where they can affect the functioning of the immune system and specific organ systems (e.g., liver, brain; Rijkers et al., 2010).

1.1.8 Sex Differences in the Gut Microbiota

Recently, Org and colleagues (2016) analyzed the gut microbiota (feces and caecum) of over 600 male and female mice from 89 strains to characterize taxa differences by sex. They found both strain-specific and sex-specific gut microbiota differences; for instance, by sex at the phylum level, Actinobacteria and Tenericutes were more abundant in males, whereas, at the genus level, *Dorea*, *Coprococcus*, and *Ruminococcus* (all from the family Lachnospiraceae) were of higher abundance in females (Org et al., 2016). Interestingly, the authors further studied sex differences in three of the mouse strains (i.e., C57BL/6J, C3H/HeJ, DBA/2J) by analyzing the overall composition in males, females, males with testes removed, females with ovaries removed, and males with testes removed and testosterone supplementation (Org et al., 2016). These groups were additionally exposed to SD or WD for eight weeks, and clear strain-, diet-, and sex-specific findings were reported (Org et al., 2016). For instance, they found that giving testectomized males testosterone returned the overall gut microbiota composition to that of intact males regardless of diet, but only in the C57BL/6J and C3H/HeJ strains (Org et al., 2016). In contrast, in C57BL/6J and DBA/2J females given the WD, ovariectomy resulted in the gut microbiota resembling that of the three groups of males, whereas intact and ovariectomized C57BL/6J and DBA/2J females did not differ in their gut microbiota composition when fed the SD (Org et al., 2016). It is crucial to reiterate that these results show that the strain of mouse, diet, and sex all resulted in clear microbiota shifts. Similar results have been

found with rats with respect to the effects of both sex and diet on shaping the gut microbiota. Indeed, S. M. Lee, Kim, Yoon, et al. (2018) demonstrated that eight weeks of 60% kcal HFD (compared to 'chow', product numbers not reported) to adolescent male and female F344 rats resulted in sex- and diet-specific shifts in the abundance of specific bacteria (e.g., *A. muciniphila* was increased with HFD feeding in both sexes, *Bacteroides* was increased with HFD only in females).

A recent review on sex differences in human gut microbiota has stated that both sex and gender are crucial factors in shaping the gut microbiota, yet "[these] factor[s] have been ignored by researchers in spite of [their] importance" (Y. S. Kim et al., 2020, p. 48). A large study with two cohorts from Europe reported on various factors that help to explain the composition of the gut microbiota (e.g., diet, stool consistency) and reports that 'gender' is also one of the covariates that explains the composition of the gut microbiota in their cohort (Falony et al., 2016). A later study by Vujkovic-Cvijin et al. (2020) confirmed the importance of sex, along with BMI, diet, age, location, and alcohol consumption, in being key potential confounding variables in studies examining the composition of the microbiota as it relates to health status. As an example, de la Cuesta-Zuluaga et al. (2019) reported with cohorts from three countries that women aged 20 to 45 years old have increased alpha diversity (i.e., having a more unique and balanced abundance of bacterial taxa; associated with better health outcomes; Hills et al., 2019), compared to men. Furthermore, with participants classified as 'normal' weight, 'overweight', or 'obese', Cuevas-Sierra et al. (2020) analyzed the presence of SNPs linked to obesity (i.e., genetic risk) and the abundance of taxa in the gut microbiota. They found that women, but not men, with more members of the family Prevotellaceae had increased genetic risk and more obesity prevalence (Cuevas-Sierra et al., 2020).

1.2 Stress Responding and Anxiety

1.2.1 Stress

Selye and Fortier (1950) and Selye (1956) formally introduced the term 'stress' in a health-related sense to describe any stimulus that leads the body to deviate from its usual

functioning and then compensate accordingly (i.e., general adaptation syndrome [GAS], from Selye, 1936). Earlier, Cannon (1929) described the concept of homeostasis or the process that regulates "the coordinated physiological reactions which maintain most of the steady states in the body…" (p. 400) but credited Bernard (1878) with the earlier idea of maintenance of a constant fluid balance in the body. The idea that the stress system and responses (i.e., GAS) can become dysfunctional, to the point of affecting normal body function, and promote disease development was first comprehensively described by Selye and Fortier (1950; i.e., "diseases of adaptation which may result from a derailment of the GAS", p. 149). However, as will be discussed in Section 1.2.3, until the discovery and characterization of the receptors for the hormones that are released as part of the stress response began in the 1960s, the GAS theory was criticized and remained incomplete (Munck et al., 1984).

The concept of 'allostasis' was first described by Sterling and Eyer (1988) to highlight that this accompanying compensation that the body undergoes during and following a response to a stressor is more complicated a conceptualization than homeostasis. To Sterling and Eyer (1988), for an organism to 'maintain stability', then it cannot undergo homeostasis because the organism needs to match its various internal physiological responses to the environment it is responding to — a process termed 'allostasis'. As later eloquently explained by Sterling (2014), to distinguish the two physiological functioning models, "…homeostasis waits for errors and then corrects them, allostasis uses prior knowledge, both innate and learned, to prevent errors and minimize them" (p. 1192). To conceptualize in another way, the definition by Thorsell (2010) states that "homeostasis refers to consistency of internal parameters within a normal range, while allostasis describes the body's way to keep stable outside the normal range by changing internal systems to match external demands" (p. 1163). In the early 1990s, McEwen and Stellar (1993) extended on the work of Sterling and Eyer (1988) on allostasis, whereby they postulated that allostasis can become problematic depending on each specific individual if the systems that regulate it are not functioning optimally — a term they called 'allostatic load'. As such, deviations from optimal functioning of these allostatic processes were broadly categorized as 'over-responding' (i.e., not stopping when the stress has concluded, responding too much) or 'under-responding' (i.e., failing to respond initially and other systems overcompensating;

McEwen & Stellar, 1993).

These early discussions of allostatic load focused on individual differences in how people react to stressors based on both behavioural (i.e., variations in the psychological categorization of the potentially stressful situation and the associated physiological responses) and biological/physiological factors (i.e., physical differences in any individual's body functioning when it encounters the stressor; McEwen, 1998; McEwen & Stellar, 1993). Today, there exist many theories of the causes and effects of stress, but in general terms, stress is a physiological response to a stimulus (a stressor) that results in the activation of the stress response system (e.g., the HPA axis; B. N. Harris, 2020). However, stressors result in the activation of other systems, such as divisions of the autonomic nervous system (e.g., sympathetic nervous system, parasympathetic nervous system) and the locus coeruleus norepinephrine system in the brainstem (Charmandari et al., 2005). Notably, these stress systems can also interact with other systems in the central nervous system and beyond. For instance, the activation of neurons that produce proopiomelanocortin (POMC) in the hypothalamus leads to the production of proteins (e.g., β-endorphins) that can inhibit the stress response and have an analgesic effect on the body (Charmandari et al., 2005).

1.2.2 The Hypothalamic-Pituitary-Adrenal Axis

In the central nervous system, the hypothalamus and brainstem are crucial for regulating the stress response. In the hypothalamus, CRF (also known as CR hormone) is released from parvocellular neurons, and arginine vasopressin (AVP, also known as antidiuretic hormone) is released from the paraventricular nucleus (PVN; Charmandari et al., 2005). In the peripheral nervous system, the HPA axis extends to the adrenal glands as part of the sympathetic adrenomedullary system and parasympathetic nervous system (Charmandari et al., 2005). A simplistic overview of a stress response, as it relates to the HPA axis, would begin in the hypothalamus, with the synthesis of CRF and AVP by specialized neurons in the PVN (S. M. Smith & Vale, 2006; Vale et al., 1981). Then, CRF is released to nearby blood vessels and travels to the anterior pituitary to bind to the CRF type 1 receptor and activate the cyclic adenosine monophosphate (cAMP) pathway, with the help of AVP (via

the vasopressin V1b receptor; S. M. Smith & Vale, 2006). In the pituitary, this activation results in the synthesis of POMC, which is a precursor for adrenocorticotropic hormone (ACTH) and other molecules (e.g., β-endorphins; S. M. Smith & Vale, 2006). Finally, ACTH is released to the blood and binds to the melanocortin type 2 receptor (MC2-R) in the adrenal glands (primarily to the intermediate layer of the adrenal cortex, the *zona fasciculata*), which, again, activates the cAMP pathway (S. M. Smith & Vale, 2006). Specifically, when ACTH binds to MC2-Rs, it activates a pathway with the intracellular secondary messenger, cAMP, that induces cAMP-dependent protein kinase A to stimulate the production and secretion of glucocorticoids, mineralocorticoids, and other adrenal steroid hormones (i.e., adrenocorticosteroids) to the blood that bind to specialized receptors throughout the body (de Joussineau et al., 2012; S. M. Smith & Vale, 2006).

As previously introduced, corticosteroids are cholesterol-derived steroid hormones that can be classified as glucocorticoids and mineralocorticoids (Taves et al., 2011). The main glucocorticoid in rats and many other animals is corticosterone (CORT); CORT is present in humans, but cortisol is the main glucocorticoid that has functional relevance during the human stress response (Kadmiel & Cidlowski, 2013; S. M. Smith & Vale, 2006). More specifically, in humans, cortisol is released into the blood from the adrenals as cortisone (i.e., inactive cortisol, bound to corticosteroid-binding globulins) and can be converted back and forth to its active form, cortisol, by the enzyme type 2 11β-hydroxysteroid dehydrogenase (Kadmiel & Cidlowski, 2013). On the other hand, mineralocorticoids (e.g., aldosterone) are released from the *zona glomerulosa* in the adrenal glands (Taves et al., 2011). For instance, aldosterone affects mineral and water reabsorption, which affects blood pressure (part of the renin-angiotensin system; Taves et al., 2011).

Mineralocorticoid receptors (MRs) can bind traditional mineralocorticoids like aldosterone, but they also have an affinity for glucocorticoids and other steroid hormones (e.g., progesterone; Taves et al., 2011). Importantly, glucocorticoid receptors (GRs) are expressed throughout the body, but even though MRs will bind glucocorticoids, they are expressed in higher numbers in specific regions (e.g., kidney, colon; Tomlinson et al., 2004). Furthermore, the binding of mineralocorticoids to MRs in such regions is promoted because the aforementioned enzyme type 2 11β-hydroxysteroid dehydrogenase converts

cortisol to cortisone and prevents cortisol from binding to MRs (Tomlinson et al., 2004). As described by De Kloet and colleagues (2005), most glucocorticoid binding to MRs occurs in the initial stages of the stress response, and only when glucocorticoid levels are high does GR binding begin to dominate to terminate the stress response. Compared to MRs, GRs have been shown to be more widely distributed in the rat brain, but MRs will bind CORT with higher affinity without added stress (Reul & De Kloet, 1986). Indeed, reduced MRs and a reduced MR to GR ratio seem to negatively affect cognition, memory, learning, and attention (reviewed by Gomez-Sanchez & Gomez-Sanchez, 2014). It has been proposed that increasing levels of MRs could have implications for the treatment of depression and borderline personality disorder (reviewed by Wingenfeld & Otte, 2019). Overall, glucocorticoids can bind to various receptors (mainly GRs, but also MRs and other G protein-coupled receptors in cell membranes; Kadmiel & Cidlowski, 2013; Timmermans et al., 2019) present in the brain and periphery (e.g., adrenal glands; S. M. Smith & Vale, 2006).

As previously mentioned, GRs are present throughout the body, almost ubiquitously (Munck et al., 1984; S. M. Smith & Vale, 2006). As reviewed by Kadmiel and Cidlowski (2013), the human GR gene (NR3C1) is comprised of 9 exons that are transcribed into mRNA and, of which, the exon 2 through 9 region is translated into protein. Alternative processing of the GR gene and post-translational modifications of the GR protein (i.e., different protein isoforms) affect the body's response (i.e., in specific cells, organs) to the glucocorticoids that are released. For example, the GRα protein, which binds glucocorticoids, results when the end of exon 8 and beginning of 9 are joined, whereas the GRβ protein, which, in part, functions to inhibit GRα, results when the end of exon 8 and downstream part of exon 9 are joined (Oakley & Cidlowski, 2011). The levels and types of GR proteins (e.g., GRα additionally has eight isoforms based on modifications in the exon 2 region) that are expressed in cells vary based on cell type and physiological state (Oakley & Cidlowski, 2011). Even at the level of the DNA sequences, polymorphisms in the human GR gene can alter the mRNA transcripts produced and have clinical implications (Kadmiel & Cidlowski, 2013). For instance, the N363S SNP results in an amino acid substitution in exon 2 and is associated with an increased risk of depressive disorders (reviewed by Kadmiel & Cidlowski, 2013; see also Gałecka et al., 2013; van

Rossum et al., 2006). In general, when glucocorticoids bind to GRs in the cytoplasm of cells, there is a conformational change in the GR which allows entry into the nucleus (i.e., GR internalization), where GR dimers can bind to glucocorticoid response elements to induce the expression of many other types of genes or transcription factors (e.g., nuclear factor κB; Oakley & Cidlowski, 2011).

1.2.3 Physiological Effects of Glucocorticoids

As reviewed by Munck and colleagues (1984), Seyle's GAS theory was unfairly and heavily criticized at the time it was postulated, especially because of the discovery that glucocorticoids (i.e., 17-hydroxy-11-dehydrocorticosterone, a glucocorticoid) could treat rheumatoid arthritis (Hench et al., 1949). However, part of Selye's theory of diseases of adaptation was that the overactivation of the stress response in the adrenal glands was the cause of many diseases, including rheumatoid arthritis (Munck et al., 1984). Because of their work on studying adrenal hormones, Hench and Kendall, along with Tadeus Reichstein, were awarded the 1950 Nobel Prize in Physiology and Medicine (Munck et al., 1984). Kendall acknowledged the contribution of Selye's idea to their theories in his speech, but that did not stop Selye's theories from being discounted at the time because he could not explain why glucocorticoids had anti-inflammatory effects (recounted by Munck et al., 1984).

Research on the discovery of GRs in the late 1960s (Munck & Brinck-Johnsen, 1968; Schaumburg & Bojesen, 1968) began to explain how glucocorticoids could both be protective against stress (i.e., helping to deal with the source of the stress by enhancing defence mechanisms) and be protective for suppressing inflammatory processes (Munck et al., 1984). Indeed, Munck et al. (1984) postulated a critical theory to reconcile these (seemingly) different ideas—that glucocorticoids might function to suppress the physiological reaction to stress by suppressing the stress response when it is time to end it (i.e., a delayed-response negative feedback loop to suppress CRF and ACTH). Along the same lines, this theory explains that, in normal physiological circumstances, glucocorticoids suppress the immune system to prevent over-responding and autoimmunity (Munck et al., 1984). Overall, in situations of stress, glucocorticoids are released and bind

to GRs throughout the body to result in various physiological responses, but at a certain point, the high levels of circulating glucocorticoids, with nowhere to bind, begin to suppress the overall system (Munck et al., 1984).

The adrenal glands release key hormones that are important regulators of physiological (e.g., aldosterone release regulates blood pressure) and psychological function (e.g., catecholamines, glucocorticoids as part of the stress response; Lyraki & Schedl, 2021). Of interest, GRs are present and function in the adrenal glands of rats (Loose et al., 1980) and humans (Paust et al., 2006). The functionality of adrenal GRs is highly relevant clinically (e.g., overexpression in adrenocortical tumours; Tacon et al., 2009). Although not often the focus of research in this area, differences in GR expression and function in adrenal glands and periphery can affect HPA axis functionality (Briassoulis et al., 2011). For instance, Cushing's syndrome results when the body has access to (e.g., from medication) or makes too much cortisol (Briassoulis et al., 2011). In the case of the body making too much cortisol, the syndrome is called Cushing's disease when it is due to excess ACTH production (e.g., from a pituitary tumour; ACTH-dependent Cushing's; Briassoulis et al., 2011). Cushing's syndrome can also refer to excess cortisol production at the source (e.g., from an adrenal gland tumour in ACTH-independent Cushing's; Briassoulis et al., 2011). Interestingly, one case report found that a young girl presented with the clinical features and symptoms of Cushing's syndrome, but in the absence of excess cortisol or ACTH and, instead, increased GRs in the periphery (in the lymphocytes; Newfield et al., 2000). There is also some evidence that adrenal GR can increase glucocorticoid release in a positive feedback loop in cellular models (e.g., in H295R cells, an adrenal cancer cell line; Asser et al., 2014), but also some debate that glucocorticoids are, instead, inhibited by adrenal GRs (e.g., in a study with male adult Sprague–Dawley rats combined with mathematical modelling; Walker et al., 2015).

As previously introduced, glucocorticoid release from the *zona fasciculata* of the adrenal glands functions as part of a negative feedback mechanism, whereby high circulating levels inhibit further release at the level of the hypothalamus and anterior pituitary (Keller-Wood & Dallman, 1984; McEwen et al., 1986; S. M. Smith & Vale, 2006). As emphasized, glucocorticoid release from the adrenal glands helps the body respond to stressors, but too

much glucocorticoid release or a stress response that is prolonged can lead to damage (e.g., immunosuppression to the point of infection; McEwen et al., 1986). In the brain, these circulating (or unbound) glucocorticoids can affect neurotransmitter and receptor levels, along with inducing cells to produce various types of enzymes and hormones (McEwen et al., 1986). Both psychological and physical stressors can stimulate the HPA axis (Mason, 1968), and a prolonged glucocorticoid response can underpin certain psychological disorders (McEwen et al., 1986). During a stress response, glucocorticoids function to break down energy stores in the body, but they also act on multiple bodily systems by binding to their various receptors (e.g., GRs, MRs) and, in turn, affect the function of a considerable number of genes and physiological processes (MacDougall-Shackleton et al., 2019). Because of their pleiotropic functionality, glucocorticoids do not function solely as stress hormones (e.g., they have metabolic and inflammatory effects), and it has been suggested that the term stress hormone be avoided when discussing glucocorticoids as it is an oversimplification of the widespread function of glucocorticoids (MacDougall-Shackleton et al., 2019).

Glucocorticoid resistance describes a physiological state where glucocorticoid binding to GRs is impaired in such a way that GRs cannot perform their function in the cells of various tissues (e.g., reduced affinity for glucocorticoids, altered transcription factor function of nuclear GR, reduced number of GRs; Chrousos, 1993). Interestingly, the body seems to compensate by increasing the activity of the HPA axis (e.g., increasing cortisol production in humans), which can result in harmful physiological consequences (e.g., fatigue, hypertension; Chrousos, 1993). It has been suggested that reduced GRs or impairments in their ability to bind glucocorticoids in the brain are central to the etiology of depression because the body compensates by releasing more glucocorticoids in response, leading to resistance (Pariante, 2006). Somewhat paradoxically, it seems that treating this proposed glucocorticoid resistance as it relates to depression can be achieved with both GR antagonists (e.g., mifepristone) and cortisol synthesis inhibitor (e.g., metyrapone), but also with GR agonists (e.g., prednisolone; Pariante, 2006). However, as explained by Pariante (2006), all of these drugs could increase the amount of cortisol (endogenously or exogenously) produced, which could increase GRs or somehow force the dysfunctional GRs to respond. In parallel, a similar story appears in the research on obesity and T2DM,

whereby acute glucocorticoid treatment normally leads to weight loss, but chronic treatment increases weight (i.e., suggesting glucocorticoid resistance; John et al., 2016). A similar pattern arises with inflammatory conditions; acute glucocorticoid treatment is used to treat autoimmune conditions, but chronic use can induce inflammation in many patients (Barnes & Adcock, 2009). In contribution to the ideas of Pariante (2006) that glucocorticoid resistance can be treated by seemingly opposite types of drugs, John et al. (2016) further suggest that understanding the reason why glucocorticoid resistance occurs could lie in increasing research on both the traditional GRα and its potential inhibitors, MR and GRβ.

1.2.4 Measuring Stress, Anxiety, and Fear

First and foremost, terms such as anxiety, stress, and fear are conceptually similar, but fear and anxiety differ from stress in subtle yet important ways. As described by Thorsell (2010), a stress response is a psychological and physiological reaction to a real or perceived threat (i.e., a stressor) that changes the external conditions of the animal (e.g., human) encountering it. Both anxiety and fear usually induce or coincide with a stress response that results in psychological and physiological effects (L. M. Shin & Liberzon, 2010), but they differ from each other based on the time course of the situation leading to the response (i.e., fear is the response to a real or perceived imminent threat, anxiety is the response to a real or perceived future threat; American Psychiatric Association, 2022). Importantly, as described in the Diagnostic and Statistical Manual of Mental Disorders (5th edition, text revision; DSM-5-TR), the behavioural and physiological responses to fear and anxiety are similar (e.g., both are related to avoidance behaviours), but fear is more linked to an autonomic response and escape behaviours, whereas anxiety is more linked to increased muscle tension and vigilance (American Psychiatric Association, 2022; Duval et al., 2015).

Human emotions such as anxiety, worry, nervousness, fear, and stress are common and often co-occurring feelings but do not always present as an anxiety disorder. These emotions can affect behaviour and are frequently short-term or situation-dependent (often referred to as state anxiety; Leal et al., 2017). On the other hand, trait anxiety is considered a stable personality trait that also increases a person's risk of demonstrating state anxiety

in specific situations (B. L. Kennedy et al., 2001). Both categories of anxiety have been linked to various mental health disorders (e.g., anxiety disorders such as generalized anxiety disorder [GAD]; other types of disorders like obsessive-compulsive disorder or major depressive disorder; Kennedy et al., 2001). However, this study by Kennedy and colleagues (2001) highlights the idea that neither state nor trait anxiety are equivalent to 'anxiety disorders' and being clear about what facet of anxiety is being measured (e.g., self-reported feelings of anxiety like worry or nervousness, specific anxiety disorders like GAD) is critical for drawing meaningful conclusions from studies.

When human fear or anxiety (e.g., worry) reach a point that they interfere with daily functioning and cause a person distress (i.e., are no longer transient fear or anxiety), the person would be diagnosed with an anxiety disorder (e.g., GAD, social anxiety disorder; DSM-5-TR; American Psychiatric Association, 2022). An anxiety response can develop from a fear response (Thorsell, 2010), and an anxiety disorder can develop when fear and anxiety responses are prolonged or mismatched (e.g., over-responsive) to the situations causing them (Duval et al., 2015; L. M. Shin & Liberzon, 2010). With this overlap of fear and anxiety, it should be noted that post-traumatic stress disorder (PTSD) is characterized as a trauma- and stress-related disorder in the DSM-5-TR and is no longer characterized as an anxiety disorder (American Psychiatric Association, 2022). While the symptom profile of PTSD overlaps with that of anxiety disorders (e.g., fear and avoidance responses), there can also be dissociative and mood-related symptoms, even without anxiety symptoms (American Psychiatric Association, 2022).

In human and rodent studies, anxiety or anxiety-related behaviours and fear have been shown to activate different brain regions. Davis et al. (2010) describe fear as 'phasic fear' in that it usually disappears quickly when the threat is gone, but anxiety as 'sustained fear' in that it is a generalized fear response that puts the brain into a prolonged apprehensive state. In male Sprague–Dawley rats, surgical lesions in the central amygdala inhibit fear responses (i.e., startle response) to an imminent threat (i.e., shock paired with light, a conditioned fear; Hitchcock & Davis, 1986). In contrast, also in male Sprague–Dawley rats, lesions in the bed nucleus of the *stria terminalis* (i.e., a structure that links the prefrontal cortex, hippocampus, and amygdala) from N-methyl-D-aspartate receptor

infusion reduces anxiety-related behaviours (i.e., acoustic startle response after administering CRF), without affecting the fear response (i.e., startle response induced through a paired light cue with shock; Y. Lee & Davis, 1997). Anxiety and fear are comparable yet distinct responses; the literature is consistently attempting to distinguish the two terms conceptually and through directed studies designed to demonstrate their differences (reviewed by Davis et al., 2010). However, how the two responses overlap with and affect the stress response is relevant for both animal and clinical human research (L. M. Shin & Liberzon, 2010). Particularly, behavioural tests designed to elicit an anxiety-related presentation (or sustained fear) in animal models seem to be more appropriate than fear elicitation for modelling human anxiety disorders (M. Davis et al., 2010).

One way to model or assay human anxiety (i.e., rodent anxiety-like or anxiety-related behaviours) is with approach-avoidant behavioural assays, such as the open field test (OFT), light-dark box (LDB), or elevated plus maze (EPM; Lezak et al., 2017). These tests measure different behavioural variables in the context of the rodent deciding to spend time in anxiety-inducing areas (e.g., center of OFT, light section of LDB, exposed and elevated arms of EPM), compared to more inherently comfortable areas (e.g., perimeter of OFT, dark section of LDB, closed arms of EPM; Lezak et al., 2017). Besides more time spent in anxiety-inducing areas and lower latency to enter these areas (both interpreted as lower anxiety-related behaviours; Lezak et al., 2017), exploratory and risk assessment behaviours (e.g., rearing, stretch-attend postures, head outs to anxiety-inducing areas, transitions between areas) can also give specific information about the state of the animal during a behavioural assay (Campos-Cardoso et al., 2023). Specifically, with the OFT, locomotor activity is commonly assessed (e.g., through manual line cross scoring, electronic tracking; Lezak et al., 2017). Specific behaviours can also be assayed (e.g., defensive behaviours) by making the environment more aversive (e.g., background noise, white light; Lezak et al., 2017).

An important consideration in research on the relationship between the stress response, anxiety-related responses, and fear responses is that the stress response (e.g., the HPA axis) can be, and is often, activated in the absence of fear or anxiety (Abelson et al., 2007). Fear and anxiety can also be induced without a concurrent HPA axis response (Abelson et al.,

2007). However, these systems do often interact; for instance, in the brainstem, as part of the locus coeruleus-noradrenergic system, there are CRF neurons in the medulla and pons (i.e., in paragigantocellular and parabrachial nuclei; Charmandari et al., 2005) that are activated after a stress response and seem to be crucial for mediating post-stress anxiety-related behaviour (McCall et al., 2015). In animal studies, the concept of exposure to novelty has been demonstrated to directly affect the HPA axis, which has been theorized to be because novel situations induce the processing of complex stimuli and require activation of the HPA axis (Herman & Cullinan, 1997). Conversely, while novelty is often anxiety-inducing to rodents, in certain situations (e.g., if there is an attractive stimulus present, especially in an area of the behavioural apparatus that is non-threatening), novelty could, in effect, be anxiolytic (Kalueff, 2006). As a whole, the concept of novelty combined with additional environmental variables can be used to study specific anxiety- or defensive-related behaviours (e.g., food presented in a novel environment after food deprivation in the novelty-suppressed feeding task [NSFT]; Lezak et al., 2017).

While behavioural assays are important for measuring responses to experimental manipulations (e.g., drug administration, stress exposure; Kalueff & Tuohimaa, 2004), the direct application of results to human disorders or symptoms should be made with caution to avoid 'overinterpretation' (Lezak et al., 2017). As outlined by Kalueff and Tuohimaa (2004), animal models are crucial for beginning to understand the causes and development of human disorders, but the fundamental knowledge that humans and animals are not equivalent on a molecular and behavioural level should always be considered. As such, caution in interpreting the cause or motivation of a certain behaviour in rodents, independent of that behaviour in humans, is key (Kalueff & Tuohimaa, 2004). For instance, rearing (i.e., a rodent standing on its hind legs) is a commonly measured exploratory behaviour; however, a recent study by Sturman et al. (2018) determined that rearing with support or no support are two distinct behaviours (i.e., unsupported rearing being more indicative of an anxiety-related presentation, supported rearing being more indicative of overall activity level). Specifically, these two behaviours were also found to vary based on the sex of the rat (i.e., females perform fewer rears of both types than males, even with similar levels of locomotion; Sturman et al., 2018). All in all, animal models are imperative for trying to understand the etiology of human conditions, but it is important to interpret

these models of human psychological states with caution, skepticism, and cognitive flexibility.

1.2.5 Molecular Changes Associated with Anxiety

The HPA axis is one of the endocrine responses to stressors, which through releasing glucocorticoids, can cause the body to use energy reserves to combat a real or perceived threat (Herman et al., 2016). Other endocrine-based responses to stress include the autonomic nervous system, glutamate system, and endogenous opioids system (see Yaribeygi et al., 2022 for a complete review). As introduced in Section 1.2.3, these glucocorticoids are critical for survival, but prolonged or inappropriate release that does not match the situation at hand can dysregulate the HPA axis and lead to physical and psychological illness (Myers et al., 2014). To conceptualize this concept in a different way, reduced functioning of the HPA axis can result from chronic overstimulation and impair appropriate stress responding (i.e., allostatic load; McEwen & Stellar, 1993) and affect the risk of psychological distress and disorders (e.g., by affecting coping strategies; Kinlein et al., 2015). For instance, Greaves-Lord et al. (2007) described that human participants who report anxiety problems had higher morning cortisol and a higher cortisol awakening response. Furthermore, in children (8 – 16 years old), self-reported anxiety was correlated with cortisol levels differentially by sex, with different types of anxiety also being associated with higher or lower cortisol at differing times of day (Kallen et al., 2008).

The number of GRs in different areas of the brain and periphery seems to be clinically relevant for various health and disease states. One early review by De Kloet et al. (1986) concluded that chronic stress, CORT administration, and ageing contribute to reducing the number of GRs in rat brains, whereas adrenalectomy increases the number of GRs. Even further, research has begun to distinguish and characterize GR functionality in altered psychological fear responding (e.g., GRs are overexpressed in PTSD; González Ramírez et al., 2020). Measuring whether GRs are present in the cytoplasm or have been internalized in the nucleus because of bound glucocorticoids can also yield information on stress-related functioning. Compared to control rats, male Sprague–Dawley rats exposed to single prolonged stress (a model of PTSD) had increased levels of cytoplasmic GRs in the dorsal

hippocampus and decreased levels of cytoplasmic GRs in the amygdala (Moulton et al., 2018). With an added fear conditioning manipulation, the control rats had increased GR internalization (to the nucleus) in cells in the amygdala; however, this GR internalization did not occur with the rats exposed to the single prolonged stress (the PTSD group; Moulton et al., 2018). The PTSD group also had reduced dorsal hippocampal cytoplasmic GRs compared to baseline levels before added fear conditioning (Moulton et al., 2018).

One molecular marker that has been linked to stress and anxiety responses in human and animal studies is brain-derived neurotrophic factor (BDNF). BDNF was the second neurotrophic factor characterized (following nerve growth factor; Barde et al., 1982) and is classified as a neurotrophin (a type of neurotrophic factor) because it is a secreted protein that acts as a growth factor or an activator for neurons (Skaper, 2012) and other non-neuronal cells (e.g., BDNF can activate astrocytes and microglia in inflammation; Ding et al., 2020). BDNF is mainly known for its role in affecting synaptic plasticity, cell survival, and neuronal growth, which can affect cognitive processes (e.g., learning and memory; Cunha et al., 2010) and behavioural responses (e.g., addiction; Lipsky & Marini, 2007). However, in rodents, there seems to be a relationship between the downregulation of BDNF and increased observed anxiety-related behaviours with exposure to social isolation stress (Murínová et al., 2017). Various types of stress in rodents (e.g., social isolation, immobilization) have also been reported to reduce hippocampal BDNF mRNA (reviewed by Duman & Monteggia, 2006). In humans, studies report that reduced BDNF is associated with anxiety symptomatology (e.g., PTSD in Dell'Osso et al., 2009; obsessive-compulsive disorder in dos Santos et al., 2011; panic disorder in Ströhle et al., 2010).

1.2.6 Stress, Anxiety, and the Gut Microbiota

A large body of research over the past couple of decades has shown that stress interferes with the development and maintenance of the gut microbiota, which can impact behaviour and health. In animals, early work with male BALB/c mice demonstrated that, compared to specific pathogen-free mice, germ-free mice had increased expression of CRF mRNA and CRF protein concentrations in the hypothalamus (which would stimulate ACTH production; Sudo et al., 2004). Additionally, in the cortex of the germ-free mice, GR

expression, of which higher GR protein levels would help to regulate the stress response, was shown to be significantly reduced compared to the specific pathogen-free mice (no difference in hypothalamus or hippocampus; Sudo et al., 2004). Relatedly, exposure to stress also seems to impair the development and composition of the gut microbiota. Infant female rhesus monkeys exposed to stress prenatally (i.e., acoustic startle test) were shown to have reduced abundance of beneficial bacteria (i.e., bifidobacteria and lactobacilli) in their microbiota (stool collected from rectal swabs; Bailey et al., 2004). Moreover, human veterans diagnosed with PTSD show distinct microbiota changes compared to veterans without PTSD, such as reduced diversity of bacterial species that comprise the microbiota, along with increased presence of pathogenic bacteria (Bajaj et al., 2019).

A study with male NMRI germ-free mice (compared to male NMRI specific pathogen-free) linked the absence of a microbiota to both reduced anxiety-related behaviours (e.g., more time in the light in the LDB, more time in the open arm in the EPM) and altered expression of related genes that are involved with synaptic plasticity (e.g., reduced BDNF mRNA in the hippocampus and amygdala; Heijtz et al., 2011). Another group demonstrated that administering antimicrobials to adult male specific pathogen-free BALB/c mice increased levels of BDNF protein in their hippocampi, reduced BDNF protein in the amygdala, and reduced observed anxiety-related behaviours in the LDB (e.g., increased time spent in light) compared to control specific-pathogen-free mice (Bercik et al., 2011). Female Swiss Webster germ-free mice have also been reported to have reduced anxiety-related behaviours (e.g., increased open-arm exploration in the EPM), increased hippocampal *Bdnf* expression, and increased CORT at baseline (Neufeld et al., 2011). On the other hand, germ-free F344 male rats (a stress-sensitive rodent strain) have been reported to have increased anxiety-related behaviours (e.g., fewer entries into the center of a novel OFT), along with higher serum CORT, increased hypothalamic CRF mRNA, and lower hippocampal GR mRNA, when compared to specific pathogen-free F44 rats (Crumeyrolle-Arias et al., 2014). Similar to humans with PTSD, distinct microbiota-related changes in humans with GAD have been reported, such as reduced diversity of the taxa that are present as part of the gut microbiota and increased levels of pathogenic bacteria compared to healthy controls (Jiang et al., 2018).

1.2.7 Effects of Diet on Anxiety

Increased or decreased anxiety-related behaviour has been reported in response to unhealthy diet administration in rodents. For instance, adult male Sprague–Dawley rats given HFD for 16 weeks (beginning in adolescence; 60% kcal fat, 20% kcal carbohydrate) or HCD control (10% kcal fat, 70% kcal carbohydrate) made fewer center entries in the OFT (no change in the EPM; Dutheil et al., 2016). In 3-month-old male Wistar rats, 13-week cafeteria diet administration (44% kcal fat, 48% kcal carbohydrate) increased observed anxiety-related behaviours in both the OFT (e.g., less time in center) and LDB (e.g., less time spent in light) compared to SD-fed rats (11% kcal fat, 63% kcal carbohydrate; González et al., 2023). Similarly, early adult female Wistar rats exposed to an early life stress paradigm had increased anxiety-related behaviours (e.g., increased latency to eat Froot Loops in the NSFT, reduced SD intake in home cage; Machado et al., 2013). In contrast, adult male C57BL/6J mice administered HFD (60% kcal fat, 20% kcal carbohydrate) starting in early adulthood had lower anxiety-related behaviour during testing if diets were administered for four weeks but increased anxiety-related behaviours with 12 weeks of diet (compared to a 10% kcal fat, 70% kcal carbohydrate control HCD; Xu et al., 2018). Interestingly, studies with longer WD or HFD diet administration have still reported reduced anxiety-related behaviours with WD exposure (e.g., 12 weeks in Demir et al., 2022; McNeilly et al., 2015). For instance, Demir et al. (2022) fed male and female Wistar rats WD (45% kcal fat, 35% kcal carbohydrate; compared to SD, 11.7% kcal fat, 67.3% kcal carbohydrate) and reported increased time spent in the open arms of the EPM (i.e., reduced anxiety-related behaviour) in the WD-fed rats.

In children aged five to ten, symptoms of anxiety, selective eating, and sensory sensitivity were found to be positively correlated; further, sensory sensitivity was found to mediate the relationship between anxiety and selective eating (Farrow & Coulthard, 2012). In young adults, high perceived stress has been reported to increase unhealthy eating patterns (in males and females; J. Choi, 2020), and the consumption of comfort foods has been shown to reduce perceived stress in women without reported depressive symptoms (Finch & Tomiyama, 2015). It has also been found that men and women exposed to acute laboratory stress consume more total calories and calories from sweet foods, which was

associated with increased state anxiety and reduced mood (compared to a control task; Rutters et al., 2009). In contrast to research on the effects of unhealthy food consumption in humans, in a recent randomized controlled trial with adults who self-reported depression, MD in combination with fish oil (compared to social therapy groups) has been reported to improve eating patterns (e.g., consumption of fewer unhealthy snacks), reduce depressive symptoms, and improve quality of life scores (Parletta et al., 2019).

1.2.8 Effects of Probiotics on Anxiety

Sudo et al. (2004) reported increased plasma ACTH, increased plasma CORT, and reduced BDNF in the cortex and hippocampus in germ-free compared to specific pathogen-free mice after restraint stress that was normalized with *B. infantis* treatment (one dose, 1×10^9 CFU). Decreased HPA activation (i.e., decreased CRF mRNA in the PVN, plasma ACTH, plasma CORT) has also been reported in response to stress in female Wistar rats given *Companilactobacillus farciminis* (1×10^{11} CFU/day), compared to a saline control group (Ait-Belgnaoui et al., 2012). Relatedly, Lacidofil® treatment (i.e., 1×10^8 CFU, two times per day during the lactation period) in Sprague–Dawley rat pups exposed to maternal separation stress improved stress-induced gut abnormalities (e.g., restoration of *Lactobacillus* species in feces; lowered stress-induced elevated CORT; Gareau et al., 2007). Furthermore, with adolescent male CD1 mice treated with *Limosilactobacillus reuteri* 23272 (i.e., 5×10^7 CFU/mL of drinking water for 12 days) during restraint stress exposure, Mackos et al. (2013) report lower anxiety-related behaviours in the OFT (i.e., more time in center) compared to vehicle-administered control mice in the groups exposed to the stressor.

This animal research can provide a starting point for which behaviours, molecular changes, and gut microbiota composition patterns may relate to human psychological health outcomes. Notably, researching how to modify the composition, function, and interaction between the microbiota and the host is theorized to be pivotal in treating mental health dysfunction (e.g., by predicting individualized pharmacological treatment success; Shoubridge et al., 2022). In a 12-week randomized, double-blind, placebo-controlled study, *Lactiplantibacillus plantarum* P8 administration resulted in a reduction of stress and

anxiety scores compared to baseline (Lew et al., 2019). In a follow-up study, the probiotic group had increased abundance of certain beneficial bacterial species in feces (e.g., *B. longum*) compared to the placebo group (Ma et al., 2021). A systematic review of randomized controlled trials that administered probiotics in humans concluded that there is still research to be done, but probiotics seem to be effective in reducing psychological symptoms like anxiety or depression in humans (Pirbaglou et al., 2016). A more recent meta-analysis that included ten randomized controlled trials on treating anxiety and depression symptoms in adults with probiotics concluded that there is evidence that probiotics can reduce symptoms of depression but not anxiety in people with diagnosed anxiety disorders, depressive disorders, or who are under stress (Chao et al., 2020).

1.3 Feeding and Metabolic Functioning

1.3.1 Obesity and the Obesogenic Phenotype

Obesity has been described as a disease where excess weight in the form of body fat impairs health (X. Lin & Li, 2021). A general and commonly accepted explanation for obesity is that the energy consumed from food exceeds how much energy is expended by the body through physiological processes and physical activity, and the body stores adipose tissue as a result (Bray, 2004). Importantly, any interpretation of this definition should consider other factors that affect obesity development and energy balance in the body (e.g., genetics, stress, financial status; see Burgio et al., 2015). Torres-Carot et al. (2022) state that if interventions to reduce energy consumption (e.g., calorie restriction) and increase energy use (e.g., physical activity) do not work in all people or do not provide lasting results, then other factors must be affecting obesity development and persistence. A recent and more detailed explanation of the energy balance model provided by K. D. Hall and colleagues (2022) highlights the theory that food intake and weight are under the control of the brain, which responds to environmental cues (e.g., types of food, stress exposure) and internal cues (e.g., food intake hormones from the gut or adipose tissue). It is suggested that environmental exposures, especially when combined with genetic predisposition, can promote internal food intake and reward signals in the brain that are not under conscious

control and require much more research to be fully understood (K. D. Hall et al., 2022). As such, it is proposed that obesity results from biological changes that promote weight gain and, fundamentally, from factors that are predominantly out of the control of any specific individual (K. D. Hall et al., 2022).

The detailed explanation of the energy balance model provided by K. D. Hall et al. (2022) highlights the importance of studying individual differences in environmental exposures and genetics that can result in obesity development, which can inform treatment and prevention strategies (e.g., a low-carbohydrate diet can result in weight loss in some but not all people). Despite difficulties in characterizing causes of excess weight, overweight and obesity are directly linked to the development of disorders such as T2DM, high blood pressure, cancer, and non-alcoholic fatty liver disease (Bäckhed et al., 2004; Bray, 2004), and excess weight can also affect mental health outcomes (Fontaine et al., 1996; Luppino et al., 2010). Relatedly, metabolic syndrome can be described as having three of a set of risk factors for cardiovascular and metabolic disease (i.e., increased waist circumference, elevated blood triglycerides, increased low-density lipoprotein cholesterol, reduced high-density lipoprotein cholesterol, hypertension, and elevated fasting glucose; Adult Treatment Panel III, 2002), but other similar definitions for metabolic syndrome exist as well (e.g., World Health Organization, 1999).

The most common way to measure obesity is through BMI, calculated by dividing weight (kg) by height squared (m^2; Aronne, 2002). BMI has the advantage of being simple and quick to use clinically and in research (Aronne, 2002). Specifically, a BMI greater or equal to 30.0 kg/m^2 is classified as 'obese' (further divided into classes I, II, and III), 25.0 or above is classified as 'overweight', 18.5 and above as 'normal', and below 18.5 as 'underweight' (Aronne, 2002). Obesity can also be measured by waist circumference (i.e., circumference measured at the midpoint between the lowest rib and top of iliac crest), waist-to-hip ratio (i.e., waist circumference divided by hip circumference at its widest point around the buttocks), waist-to-height ratio (i.e., waist circumference divided by height), and newer methods as well (e.g., body roundness index, visceral adiposity index; Al-Shami et al., 2022). In fact, waist circumference and waist-to-hip ratio have been shown to be better predictors of T2DM presence than BMI, whereas waist-to-height ratio is a better

predictor of hypertension than BMI (J. R. Choi et al., 2018). For assessment of excess body fat, a recent systematic review and meta-analysis on the performance of tools for measuring obesity concluded that there are concerning limitations with both BMI and waist circumference, along with a lack of evidence for both waist-to-hip ratio and waist-to-height ratio (Sommer et al., 2020). Nevertheless, obesity is a risk factor for metabolic syndrome and clinically, these clinical tools, such as BMI and waist circumference, are important for assessing risk of obesity-related health consequences (Sperling et al., 2015).

A common way to measure obesity in rodent studies is by examining body weight or weight gain of experimental animals compared to a control group (i.e., if experimental animals are significantly higher in weight, then obesity is assumed to result; Novelli et al., 2007). Body weight is a quick, minimally invasive, and commonly used obesity indicator that allows for tracking over time within the same study and comparability between studies. Nonetheless, incorporating additional factors with association to body weight is important for characterizing obesity (e.g., calorie consumption, lipid and hormone levels in blood; Novelli et al., 2007). For example, plasma leptin levels are highly positively correlated with body weight in rodents and can be a marker of obesity (Maffei et al., 1995). Other than leptin, other indicators of obesity in rodent studies are blood glucose responses, insulin levels, triglyceride levels, and fatty acid levels (reviewed by Buettner et al., 2007; Rosini et al., 2012). More sophisticated measures of body composition that examine factors such as total body fat, lean tissue mass, and bone content have also been used to characterize obesity in animal research (Engelbregt et al., 2001). Other indicators of obesity can be studied by measuring body composition changes, fat mass increases (e.g., white adipose tissue is increased with obesity), and energy expenditure (e.g., heat production directly with a calorimeter, chamber measurements of O_2 and CO_2; reviewed by Tschöp et al., 2012). A rat version of the BMI (i.e., body weight in grams divided by body length in square centimeters) has also been used to infer body fat mass in combination with measuring serum leptin levels (Engelbregt et al., 2001).

1.3.2 Overview of Food Intake and Hormones

Various peripherally produced hormones (e.g., from adipose tissue, the pancreas, the gut) regulate food intake and energy balance in the body by acting on hypothalamic brain regions (e.g., the arcuate nucleus [ARC], the PVN, the lateral hypothalamus, the dorsomedial hypothalamus, the ventromedial hypothalamus) and the brainstem (e.g., the *nucleus tractus solitarii*), directly or via the vagus nerve (Stanley et al., 2005). Peripheral adipose tissue is the major source of the anorexigenic (i.e., appetite-reducing) hormones adiponectin, resistin, and leptin (Stanley et al., 2005). As well, although pancreas-produced insulin (from beta cells) and glucagon (from alpha cells) have opposing functions with respect to regulating blood glucose levels (i.e., insulin reduces, and glucagon increases, blood glucose), they are both anorexigenic factors to the brain and liver (Moede et al., 2020; Woods et al., 2006). Specifically, insulin is a signal for the liver to reduce glucose production while also telling the hypothalamus to reduce energy expenditure (Woods et al., 2006). In comparison, glucagon release stimulates the liver to produce glucose from glycogenolysis or gluconeogenesis (Moede et al., 2020).

Anorexigenic (i.e., appetite-reducing) hormones produced mainly in the gut include but are not limited to, peptide YY, oxyntomodulin, pancreatic polypeptide (also produced in the pancreas), glucagon-like peptide 1 (GLP-1), and cholecystokinin (Al-Massadi et al., 2019; Perry & Wang, 2012; Stanley et al., 2005). It should be noted that these hormones, while categorized here as anorexigenic, also have critical physiological functions in the periphery (e.g., GLP-1 reduces gastric motility in the gut; cholecystokinin increases bile and acid secretions in the gut; Alhabeeb et al., 2021). In the periphery, GLP-1 is critical for regulating glucose levels in the blood through direct interactions with both insulin and glucagon (i.e., stimulating insulin, inhibiting glucagon release; Woźniak et al., 2021), but GLP-1 is also produced in the brainstem and specific hypothalamic regions (Stanley et al., 2005). Centrally, in the ARC, specialized POMC-expressing neurons can be stimulated by peripheral hormones (e.g., leptin, serotonin) to produce POMC and related compounds like ACTH, β-endorphins, and α-melanocyte-stimulating hormone to increase energy use and reduce food intake (Schulz et al., 2010; Toda et al., 2017).

In contrast, orexigenic (i.e., appetite-stimulating) hormones increase food intake, with ghrelin in the periphery and neuropeptide Y (NPY), agouti-related peptide (AgRP), and orexin-A in the brain (Beck, 2006; Chieffi et al., 2017; Ilnytska & Argyropoulos, 2008). In the ARC, NPY/AgRP-expressing neurons increase food intake and reduce energy use through various mechanisms, including through the release of gamma-aminobutyric acid (GABA) to inhibit POMC-expressing neurons and by acting on melanocortin 4 receptor-expressing neurons in the PVN that promote food intake (Cowley et al., 2001; Huang et al., 2021). If ghrelin is injected into the hypothalamus, especially the ARC, food intake is stimulated and weight gain results, which is proposed to be due to binding to NPY-AgRP neurons that contain ghrelin receptors (shown with adult male Wistar rats; Wren et al., 2001). Within the hypothalamus, other specialized regions and neuropeptides can also regulate food intake (e.g., BDNF administration reduces food intake; Gotoh et al., 2013; ChuanFeng Wang et al., 2007) through interactions with POMC-neurons (Stanley et al., 2005; Toda et al., 2017). NPY-expressing and POMC-expressing neurons are also present in the brainstem and interact with cells in the PVN as part of food intake regulation (Stanley et al., 2005).

Leptin is a 167-amino acid anorexigenic peptide hormone produced mainly by white adipose tissue (Obradovic et al., 2021) and was first characterized by Yiying Zhang and colleagues (1994). As previously stated, leptin acts on anorexigenic POMC neurons in the ARC by stimulating action potentials of these POMC neurons but also inhibiting the orexigenic NPY neurons and GABA neurons (Cowley et al., 2001). Functionally, leptin release reduces food intake and body weight to a certain point (i.e., until leptin resistance occurs), and it is well-established that leptin is not an effective treatment for obesity because of leptin resistance (A. G. Izquierdo et al., 2019). In metabolic research, leptin resistance is often inferred with the presence of both elevated leptin and evidence of obesity (C. Yamada, 2021). Of note, intracerebroventricular leptin injection reduces body weight and food intake in female Sprague–Dawley rats, more so in rats that have had their adrenal glands removed (Zakrzewska et al., 1997). Interestingly, administering dexamethasone (a synthetic glucocorticoid) subcutaneously has been shown to inhibit the anorexigenic effects of leptin (Zakrzewska et al., 1997). These findings suggest that there is an

interaction between the stress response and the release of leptin, which has implications for the development of leptin resistance in situations of acute and chronic stress.

Ghrelin is a peripherally produced 28-amino acid orexigenic hormone first described by Kojima and colleagues (1999). While ghrelin is mainly produced in the stomach (gastric fundus, by X/A-like cells; Date et al., 2000; Kojima et al., 1999) and pancreas (Andralojc et al., 2009), it has also been reported to be produced by other peripheral organs, such as the kidneys (Mori et al., 2000) and placenta (Gualillo et al., 2001). The exact mechanisms of action of ghrelin on the brain are still being actively studied, but it has been proposed that circulating ghrelin enters the brain through blood-brain barrier vessels and binds to both POMC and NPY neurons in the hypothalamus to increase food intake (i.e., inhibits POMC-neurons, stimulates NPY/AgRP neurons; Cowley et al., 2001, 2003; Schaeffer et al., 2013). There is speculation that ghrelin might be produced in the hypothalamus as well, but as stressed by Fernandez et al. (2016), it is difficult to distinguish between ghrelin that would be produced in the brain and ghrelin that is entering the brain via circulation. Ghrelin is most commonly known as a peripherally produced orexigenic peptide that is found in lower amounts in people with obesity (Y. Wang et al., 2022) and in higher amounts in people with anorexia nervosa (Otto et al., 2001). For instance, human work has shown that women with obesity have lower ghrelin before and after eating compared to women in the 'normal' BMI category (Kiessl & Laessle, 2017). Similarly, Rouach et al. (2007) found higher baseline plasma ghrelin concentrations in women with a 'normal' BMI compared to women who were in the 'obese' category.

Once released into the bloodstream from the stomach and other peripheral organs, ghrelin exists as acylated ghrelin (or acyl ghrelin) and, more often, as deacylated ghrelin (or unacylated, des-acyl, nonacylated ghrelin; Y. Wang et al., 2022). Acylated ghrelin exerts effects peripherally and centrally through a receptor known as the ghrelin receptor (or the growth hormone secretagogue receptor) and is commonly referred to as active ghrelin because of its ability to bind to this receptor (reviewed by Delhanty et al., 2014). Until recently, the physiological function of deacylated ghrelin has remained largely unknown. Of note, Fernandez et al. (2016) showed that in wild-type C57BL/6J mice, deacylated ghrelin binds to neurons (e.g., NPY-expressing neurons) in the ARC, but this binding in

the ARC was also observed in mice without ghrelin receptors. These findings suggest that binding is also occurring via another receptor type that remains uncharacterized (Fernandez et al., 2016). Interestingly, in the wild-type mice, central (intracerebroventricular) administration of deacylated ghrelin reduced food intake that was increased after administration of acylated ghrelin, but only when acylated ghrelin was administered peripherally (i.e., no change in food intake by deacylated ghrelin with prior central acylated ghrelin administration), suggesting a potential antagonistic role of deacylated ghrelin on acylated ghrelin at the ghrelin receptor in the ARC (Fernandez et al., 2016). Indeed, a recent review on the function of ghrelin suggests that finding and characterizing the receptors for deacylated ghrelin could be critical in understanding the vast physiological effects of ghrelin (Deschaine & Leggio, 2022).

NPY was first characterized by Tatemoto and colleagues (1982) as a 36-amino acid orexigenic peptide that is part of the pancreatic polypeptide family (Beck, 2006; Reichmann & Holzer, 2016). NPY is expressed in the hypothalamus and adrenal medulla (by chromaffin cells; Gupta et al., 2017), but NPY is also part of the enteric nervous system as a regulator of digestive processes (e.g., inhibits gastrointestinal motility; Holzer et al., 2012). The administration of NPY to male C57BL/6J mice and Sprague–Dawley rats results in increased food intake, body weight, and fat, alongside increased plasma leptin, insulin, and CORT (Raposinho et al., 2001). Moreover, increased NPY expression and plasma protein levels in response to different types of chronic stressors and WD stimulates leptin secretion and fat storage in mice (adult male 129SvJ strain; Kuo et al., 2007). On top of NPY playing a role in the induction of leptin resistance, leptin also seems to oppose the effects of NPY (Furness et al., 2001; Schwartz et al., 1996). Even further, vagotomy in female Sprague–Dawley rats results in weight loss, reduced food intake, and increased NPY (Furness et al., 2001). Interestingly, NPY expression in adult male and female *ob*/*ob* mice has recently been reported to be tissue-dependent and distinct in each sex (Werdermann et al., 2021). However, another study reported that fasting in both sexes of mice increased NPY mRNA in the hypothalamus and adrenal glands (Chua et al., 1991), suggesting that sex differences in NPY expression or protein levels could depend on the physiological state (e.g., hunger status, time of measurement) of the experimental subjects.

One difficulty in studying and conceptualizing metabolic hormones is that some anorexigenic hormones (e.g., leptin) are found to be increased with obesity, whereas some orexigenic hormones are found to be reduced (e.g., ghrelin; Montégut et al., 2021), but this is not always the case. With leptin, resistance (i.e., reduced response to leptin-associated appetite-reducing signals) is a consequence of obesity, even though leptin and leptin receptor deficiency has been shown to be a cause of obesity (Stanley et al., 2005; Wasim et al., 2016). In line with these findings, weight loss has been shown to lead to the reduction of circulating anorexigenic hormones (e.g., leptin, peptide YY, cholecystokinin, insulin) and to increase circulating levels of the orexigenic hormone, ghrelin (Sumithran et al., 2011). Concerning energy expenditure, a low metabolic rate has been shown to be associated with higher ghrelin and lower insulin in adult women (Hajishizari et al., 2022). However, anorexigenic peptides like pancreatic polypeptide and peptide YY have been reported to be reduced with obesity, in contrast to other anorexigenic peptides like leptin and insulin (Montégut et al., 2021; Sumithran et al., 2011). As well, leptin has been reported to both induce and improve insulin resistance, which is proposed to be related to leptin affecting insulin release from the pancreas (Ceddia et al., 2002), even though both insulin and leptin are increased in people with obesity (R. Kumar et al., 2020), and obesity is a cause of both insulin and leptin resistance (Könner & Brüning, 2012).

Although leptin and insulin have anorexigenic effects in the brain, receptors for both are found on various types of neurons throughout the hypothalamus and brainstem that have both orexigenic and anorexigenic functions (e.g., on both POMC-expressing and NPY/AgRP-expressing neurons in the ARC; Belgardt & Brüning, 2010). As summarized by Könner and Brüning (2012), in lean mice that are responsive to insulin or leptin, the anorexigenic signals are interpreted properly by POMC-expressing neurons in the ARC, which activates a pathway to reduce food intake. However, when these neurons are unresponsive to insulin, food intake is not suppressed, and obesity can persist. Importantly, increasing our understanding of the time course and consequences of innate immune system-associated inflammation in the hypothalamus, along with the action of non-neuronal (i.e., glial) cells in this region, has been suggested to help disentangle the complex relationship between metabolic hormones, food intake, and obesity development and persistence (Thaler et al., 2010). In sum, characterizing peripheral and central control of

food intake is complex and dependent on a variety of concurrent physiological variables that can be altered with conditions such as obesity.

1.3.3 Links Between Stress, Anxiety, and Feeding

It was well-known by the mid-1980s that glucocorticoids opposed the action of insulin, although there was debate as to why this was beneficial for an organism (i.e., either because glucocorticoids mobilized glucose to help the organism deal with the stress or because they directly suppressed insulin so that hypoglycemia did not result; Munck et al., 1984). A body under stress is also affected at the level of appetite control in the hypothalamus, whereby CRF functions as an anorexigenic factor that interacts with both NPY and leptin (Charmandari et al., 2005). Recent work has established that stress and T2DM are positively associated through multiple potential behavioural and molecular mechanisms (e.g., lack of physical activity, stress-induced insulin resistance; reviewed by Lloyd et al., 2005; Yaribeygi et al., 2022), and stress might be a risk factor for later development of T2DM (Mooy et al., 2000). In humans, one recent systematic review and meta-analysis concluded that healthy participants with normal glucose tolerance who were administered glucocorticoids (i.e., dexamethasone, prednisolone) had higher fasting plasma glucose, plasma insulin, and insulin resistance after administration (P.-Z. Zhou et al., 2016).

NPY is a potent orexigenic factor, but it is also well-established for its role in aiding with stress resilience (H. Cohen et al., 2012; Hawley et al., 2010; Heilig, 2004) and reducing the presentation of anxiety-related behaviours (Heilig, 2004; Sajdyk et al., 2004) in animal models. Further, Asakawa et al. (2003) showed that administering leptin intraperitoneally to *ob*/*ob* C57BL/6J mice (sex of animals not reported) that cannot produce leptin, compared to *ob*/*ob* mice given saline, resulted in lower anxiety-related behaviours observed in the EPM (e.g., more entries into the open arms). A later study similarly showed that male *ob*/*ob* C57BL/6J mice displayed more anxiety-related behaviours in the LDB (i.e., less time spent in light, fewer transitions) than lean littermate controls (Finger et al., 2010). In human males, plasma levels of NPY have been shown to increase, alongside cortisol and norepinephrine, in response to stress (i.e., before and after military training; Morgan et al., 2001). In contrast, females exposed to intimate partner violence had reduced cortisol and

no change in NPY compared to control females, and neither cortisol nor NPY levels were correlated with PTSD symptoms (Seedat et al., 2003).

In rodent studies, the orexigenic peptide ghrelin also seems to play a role in the complex relationship between stress, anxiety-related behaviours, and feeding hormones. However, whether ghrelin is anxiogenic or anxiolytic is still being elucidated. Ghrelin has been shown to have anxiolytic effects when administered peripherally prior to OFT and EPM testing; ghrelin reduced anxiety-related behaviours, such as increased time spent in the center or open arms in early adult male NMRI mice (Jensen et al., 2016). Ghrelin knockout mice have more anxiety-related behaviour after acute stress, alongside reduced CORT that is increased when the knockout mice are administered ACTH (Spencer et al., 2012). Adolescent male mice (C57BL/6J) exposed to novelty stress (i.e., single housing in a new cage) had reduced food intake, reduced NPY mRNA in the hypothalamus, and reduced plasma ghrelin three hours post-stress, with increased serum CORT 30 mins post-stress, compared to mice not exposed to stress (Saegusa et al., 2011). In this study, administering both ghrelin and a ghrelin receptor antagonist reversed the decrease in food intake after the novelty stress (Saegusa et al., 2011). However, other work has shown that intracerebroventricular injection of ghrelin to early adult male Wistar rats increases freezing behaviours in the OFT and reduces exploration in anxiety-inducing areas of the OFT and EPM (Carlini et al., 2002). Further, compared to sham stress, plasma ACTH and ghrelin were increased following one-hour acute stress exposure (i.e., water avoidance stress) in two strains of adult female rats (i.e., stress hyper-responsive Wistar Kyoto rats and Sprague–Dawley rats; Kristenssson et al., 2006).

Overall, ghrelin seems to function as more than just an agent to induce food intake (reviewed by Pradhan et al., 2013), and it has been consistently reported to affect stress responding and anxiety presentation in humans as well. Ghrelin, but not leptin, has been reported to increase following an acute social laboratory stress that increased self-rated anxiety in male and female participants compared to the no-stress participants (McKay et al., 2021). Again, ghrelin, but not leptin, has also been reported to be increased in children (6 – 12 years old) with anxiety disorders compared to healthy control children (Ozmen et al., 2019). Ghrelin has been reported to be increased in humans with anxiety disorders and

adults with prior exposure to childhood trauma and current symptoms of binge and emotional eating (Rossi et al., 2021). In fact, a broad regulatory role of ghrelin in HPA axis responding and associated anxiety continues to be suggested (Asakawa, Inui, Kaga, Yuzuriha, Nagata, Fujimiya, et al., 2001; Chuang & Zigman, 2010), with ghrelin even being referred to as a stress hormone that has multiple functions depending on participant and study characteristics, such as the context of the stress and hunger state (Stone et al., 2020; Wittekind et al., 2022). For instance, in postmenopausal women with symptoms of depression, increased acylated ghrelin is related to increased BMI and increased self-reported depressive symptoms, but it remains unknown if ghrelin is a cause or consequence of poorer mental health and increased BMI (Naufel et al., 2021).

1.3.4 Metabolic Functioning and the Gut Microbiota

An obesogenic phenotype has environmental and genetic influences, but it has also been reported that the composition of the human gut microbiota is heavily involved in the development of obesity (e.g., individuals with obesity have lower bacterial species diversity; Le Chatelier et al., 2013). Studies have shown that the presence and composition of the gut microbiota directly affects host energy use and storage. Namely, Bäckhed et al. (2004) provided adult germ-free male B6 mice with a cecal microbiota from conventional animals and reported a dramatic increase in body fat alongside reduced food intake that mirrored that of conventional mouse donors. Some studies report that obesity is associated with increased presence of the Firmicutes phylum and decreased presence of the Bacteroidetes phylum and that the ratio of Firmicutes to Bacteroidetes decreases with weight loss (Koliada et al., 2017; Ley et al., 2005, 2006). Nevertheless, some researchers also caution against the use of the Firmicutes to Bacteroidetes ratio (e.g., Guinane & Cotter, 2013; Koliada et al., 2017) and have further reported that obesity may be linked to the presence or absence of certain more specific taxa, such as decreased abundance of the genus *Bifidobacterium* (Guinane & Cotter, 2013).

Even with research on which taxa might influence the development of obesity and impair or improve metabolic functioning still ongoing, it is recognized that the overall gut microbiota has been shown to directly affect metabolism in hosts (e.g., processing

nondigestible carbohydrates; Turnbaugh, Ridaura, et al., 2009). Indeed, by helping to produce vital compounds for the host (e.g., short-chain fatty acids [SCFAs], bile acids, vitamins), microbes in the gut can directly affect host health outcomes (Singh et al., 2023). Interestingly, higher serum ghrelin in male Sprague–Dawley rats has been associated with reduced abundance of *Bifidobacterium* and *Lactobacillus* species, whereas the opposite relationship has been reported for leptin and these taxa (Queipo-Ortuño et al., 2013). In addition, germ-free C57BL/6J male mice have been reported to have increased expression of mRNA for proteins linked to reduced obesity risk in the hypothalamus (i.e., BDNF) and brainstem (i.e., BDNF, GLP-1 precursor), alongside reduced expression of other anti-obesity peptides (e.g., POMC) and increased expression of pro-obesity peptides (e.g., NPY; Schéle et al., 2013). Moreover, in this study, conventional mice were found to not respond as much to leptin administration (intraperitoneal injection) when compared to germ-free mice, who had significant weight loss and a reduction in both NPY and AgRP after three days of treatment compared to their baseline levels (Schéle et al., 2013). Overall, it is apparent that the gut microbiota is a critical aspect of metabolic health that interact with host genetics and environmental factors (e.g., nutrition) to affect the risk of developing obesity-related diseases such as T2DM (Reimer, 2019; Ryan & Delzenne, 2016).

1.3.5 Effects of Diet on Metabolic Functioning

As introduced in Section 1.1.6, diet is a robust determinant of gut microbiota composition, and dietary factors, such as increased fibre consumption, have been shown to affect levels of metabolites produced from gut microbes and, in turn, affect metabolic disease development (Dabke et al., 2019). In male and female C57BL/6J mice, WD administration (45% kcal fat, 35% kcal carbohydrate) starting in adolescence has been shown to increase body weight, food intake, and fat mass in both sexes, compared to HCD control (10% kcal fat, 70% kcal carbohydrate), depending on the length of WD administration (e.g., body weight increased in males after 14 weeks, but after 27 weeks in females; Y. Yang et al., 2014). As well, in C57BL/6J mice (sex not reported), administration of an HFD (60% kcal fat, 20% kcal carbohydrate), compared to SD (13% kcal fat, 56% kcal carbohydrate), results in increased total daily calorie intake and feeding outside of regular feeding

windows (i.e., during the light part of the light-dark cycle; Licholai et al., 2018). In another study, cafeteria diet-fed (44% kcal fat; 48% kcal carbohydrate) male Wistar rats showed more weight gain, calorie intake, and unhealthy metabolic parameters (e.g., higher triglycerides) compared to SD-fed rats (11% kcal fat, 63% kcal carbohydrate; González et al., 2023). Also compared to SD-fed rats, rats administered an HFD (60% kcal fat, 20% kcal carbohydrate) for eight weeks starting in adolescence had increased plasma glucose and total cholesterol (J.-H. Shin et al., 2018). Interestingly, in the previously mentioned study (see Section 1.2.7) by Machado et al. (2013), the early adult female Wistar rats exposed to early life stress consumed less SD and gained less weight from weaning than control rats, but when given the choice to eat a WD or SD (kcal% not provided for diets), the females exposed to early life stress consumed more of the WD than the non-stressed controls; yet, they still gained less weight than controls with the four-week exposure to WD. In non-human primates (female crab-eating macaque), administering a WD formulation matched to a human WD for approximately 30 months results in increased body fat, energy expenditure and activity levels, insulin resistance, and increased liver fat deposits compared to baseline (Shively et al., 2019). Additionally, giving a separate group of monkeys an MD reduced triglyceride levels compared to baseline (Shively et al., 2019).

As complemented by the animal studies in the previous paragraph, diet in humans can have vast effects on behaviour and physiology. When human fecal samples are transplanted to germ-free C57BL/6J male mice and left to colonize, microbial communities change rapidly (within one day) in response to a sudden shift from an SD to a WD (Turnbaugh, Ridaura, et al., 2009). Thus, in male mice that were initially germ-free and would be protected from the obesogenic consequences of a WD (e.g., Rabot et al., 2010), the presence of a gut microbiota harvested from humans was able to promote unhealthy consequences (e.g., increased adiposity) from the diet (Turnbaugh, Ridaura, et al., 2009). Correspondingly, in participants with T2DM, bacteria that produce SCFAs are increased by dietary fibre intake and linked to improved hemoglobin A1c levels (i.e., a measure of the three-month average blood sugar; L. Zhao et al., 2018). Moreover, in postmenopausal adult women in the 'obese' BMI category given six weeks of daily probiotic (*Lacticaseibacillus paracasei* F19, 9.4×10^{10} CFU/day), flaxseed, or placebo, it was reported that only flaxseed improved markers related to insulin resistance (Brahe et al., 2015).

1.3.6 Effects of Probiotics on Metabolic Functioning

In a meta-analysis of the effects of probiotics on various metabolic parameters in human adults, probiotics, in general, were found to lead to significant reductions in body weight, BMI, waist circumference, and body fat (Z.-B. Wang et al., 2019). Probiotics can improve obesogenic outcomes in rats (e.g., female Sprague–Dawley rats given *Lactiplantibacillus plantarum* in Karlsson et al., 2011; *Bifidobacterium* species to male Sprague–Dawley rats in An et al., 2011; Yin et al., 2010; *L. helveticus* R0052 to an IL-10 deficient mouse model in Ohland et al., 2013). The abundance of one species of bacteria, *A. muciniphila*, has been reported to be decreased in the caecal contents of adult male C57BL/6 mice with genetic obesity (i.e., leptin-deficient, *ob*/*ob*) and HFD-induced obesity (Everard et al., 2013). As well, the administration of *A. muciniphila* or a prebiotic that increases its abundance (i.e., oligofructose) improved various metabolic measures in HFD-fed mice (e.g., reduced total fat mass, insulin resistance; Everard et al., 2013). In the previously mentioned study (see Section 1.3.5) by J.-H Shin and colleagues (2018) that administered an HFD to male Sprague–Dawley rats and measured various metabolic parameters, the authors further showed that the addition of probiotic treatment (Duolac Gold; combination of *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* species; 1×10^7 CFU/day) improved poor metabolic outcomes that were induced by the diet administration (e.g., whole fat and abdominal fat volume, serum triglyceride levels).

Probiotics comprised of species of *Lactobacillus* have been shown to be beneficial for decreasing low-density lipoprotein (e.g., *Lactiplantibacillus plantarum* to men with elevated total cholesterol in Bukowska et al., 1997) and improving insulin sensitivity (e.g., *L. acidophilus* NCFM to men with T2DM in Andreasen et al., 2010). Further, administration of *L. gasseri* SBT2055 provided in fermented milk for 12 weeks (approximate dose of 5×10^{10} CFU, twice daily) to participants mainly in the 'overweight' BMI category leads to the reduction of adipose mass, overall body weight, BMI, and waist and hip size, compared to participants consuming the milk without the SBT2055 strain (Kadooka et al., 2010). *Bifidobacterium* probiotics have also been reported to improve metabolic function in humans. Bernini et al. (2016) report reduced BMI, total cholesterol, low-density lipoprotein, and plasma TNF-α and IL-6 from baseline after 45 days of

treatment with *B. lactis* HN019 (2.72×10^{10} CFU per day in fermented milk). Furthermore, combination *Bifidobacterium* and *Lactobacillus* probiotics have been reported to benefit people, especially when combined with dietary intervention. For instance, women in the 'overweight' or 'obese' BMI category consumed a 6-strain probiotic combination (including species of *Lactobacillus*, *Lactococcus,* and *Bifidobacterium*) or placebo (i.e., randomized, double-blind, placebo-controlled) for eight weeks in combination with a prescribed dietary intervention (A. C. Gomes et al., 2017). Compared to the placebo group, those in the probiotic group saw greater benefits from the dietary intervention on markers of metabolic health (e.g., reduction in waist circumference; A. C. Gomes et al., 2017). It should be noted that there is evidence for probiotic treatment improving metabolic parameters in humans, but there seem to be strain-specific effects for the impact of probiotics on such parameters. For instance, despite various probiotics being reported here to improve metabolic health, as mentioned above (see Section 1.3.5), Brahe et al. (2015) reported that the six-week administration of *Lacticaseibacillus paracasei* F19 did not affect measured metabolic parameters.

1.4 IMMUNE RESPONDING

1.4.1 The Immune System

The immune system, or immunity, can be first divided into innate responses for the immediate or reactive defence to pathogens (e.g., neutrophil recruitment to the site of infection, cytokine release from macrophages), compared to the more thorough and longer-term, adaptive responses (e.g., T cell or B cell responses; Parkin & Cohen, 2001). The immune system refers to specific cells, chemical messengers, and proteins that function to protect bodily organs and systems (e.g., from microbes, toxins, cancer); this system relies on crosstalk and cross-functionality between innate and adaptive immune responses (Marshall et al., 2018). Non-specific innate responses could include a quick response of leukocytes (e.g., macrophages) to destroy pathogens or an increase in body temperature (i.e., fever) to prevent the growth of microbes (Marshall et al., 2018). As part of the adaptive immune system, lymphocytes are a type of leukocyte that produce antibodies in

response to pathogens (i.e., B lymphocytes or B cells; humoral or antibody-mediated response) or direct the function of other leukocytes, such as macrophages, and produce proteins in response to infection (i.e., T lymphocytes or T cells, helper or killer T cells; cell-mediated immune response; Parkin & Cohen, 2001).

In response to infection, B cells, produced from hematopoietic stem cells in the bone marrow, differentiate into cells known as plasma cells (Marshall et al., 2018). B cells have specific antigen-binding receptors on their cell membranes that recognize foreign invaders and produce antibodies in response (Marshall et al., 2018). B cells can also differentiate as memory B cells, which exist for a period of time with the antigen-binding receptor ready to respond to a subsequent infection of the same or similar type (Marshall et al., 2018). Alternatively, T cells are produced from hematopoietic stem cells in the bone marrow but migrate and mature in the thymus (Marshall et al., 2018). T cells contain specific T-cell receptors (TCRs) in their cell membranes and work closely with members of the innate immune system (i.e., antigen-presenting cells [APCs], e.g., macrophages, dendritic cells), and sometimes B cells, in antigen recognition (Marshall et al., 2018). APCs have cell-surface proteins called the major histocompatibility complex (MHC) that are classified as Class I (i.e., that present intracellular peptides) or Class II (i.e., that present extracellular peptides) to match up to specific T cells (with specific TCRs) to secrete specific protein messengers (e.g., cytokines) in response to this pathogen recognition (Marshall et al., 2018). One major group of protein messengers produced by cells of both the adaptive (e.g., T cells) and innate (e.g., macrophages, neutrophils) responses are cytokines, which, when released, affect the response of other cells (Parkin & Cohen, 2001).

1.4.2 Cytokines

Cytokines can be categorized as interleukins (IL-; produced by leukocytes and affect other leukocytes; Akdis et al., 2011), chemokines, colony-stimulating factors, interferons (i.e., interfere with viral replication or modulate immune responses; e.g., IFN-γ), tumour necrosis factors (i.e., TNFs; Parkin & Cohen, 2001; Paul & Seder, 1994; Wallach, 2018), and growth factors (e.g., vascular endothelial growth factor [VEGF]; Holmes & Zachary, 2005). Chemokines (e.g., macrophage chemoattractant protein-1 [MCP-1]; macrophage

inflammatory protein [MIP]-1α; MIP-3α; regulated on activation, normal T-cell expressed and secreted [RANTES]; growth-regulated oncogene/keratinocyte-derived chemokine [GRO-KC]) are types of cytokines that help direct the movement of various leukocytes (e.g., neutrophils, macrophages, lymphocytes like B cells and T cells; Griffith et al., 2014; Parkin & Cohen, 2001). Colony-stimulating factors affect stem cells and the growth of cells like myeloid cells (i.e., granulocyte colony-stimulating factor [G-CSF]), macrophages (i.e., monocyte colony-stimulating factor [M-CSF]), and monomyelocytic cells (i.e., granulocyte-macrophage-colony stimulating factor [GM-CSF]; Parkin & Cohen, 2001).

When T cells encounter an MHC that activates their TCRs to release cytokines, they are induced to differentiate into cytotoxic T cells (CD8+ T cells) or T-helper cells (CD4+ T cells; Marshall et al., 2018). Cytotoxic T cells result from TCRs encountering APCs with the Class I MHC (Marshall et al., 2018). In contrast, T helper cells (i.e., T cells that have CD4 surface receptors that recognize the Class II MHC; Marshall et al., 2018) are responsible for much of the cytokine production in the body and can be divided into Th1 (T helper) and Th2 depending on the cytokines produced (i.e., Th1-type and Th2-type cytokines; Berger, 2000). More specifically, most cytokines can be categorized as pro-inflammatory (usually Th1-type; e.g., IFN-γ, IL-1β, IL-2, IL-12, TNF-α, GM-CSF), which activate the immune system in response to foreign invaders, or anti-inflammatory (Th2-type; e.g., IL-4, IL-5, IL-10, IL-13), which counteract pro-inflammatory mechanisms (Berger, 2000; H. L. Lee et al., 2019). It should be noted that other categories of Th cells release specific cytokines (i.e., Th17, Th22, Th9, regulatory T cells; Raphael et al., 2015). Interestingly, regulatory T cells (Tregs, a type of CD4+ T cell) seem to suppress the immune system and cytokine production, which is critical for the treatment of allergies and autoimmune diseases (Kondělková et al., 2010).

At a basic level, Th1-type cytokine overproduction can lead to autoimmune diseases, whereas Th2-type cytokine overproduction has been linked to allergic responses and cancer development (Berger, 2000; H. L. Lee et al., 2019). In reality, categorizing Th cells based on which cytokines they produce is sometimes too simplistic, and it should be noted that cytokines deemed anti-inflammatory or pro-inflammatory can have both functions depending on the context (Berger, 2000; H. L. Lee et al., 2019). For instance, IFN-γ can

increase macrophage activity (Raphael et al., 2015) and increase the immune response to infection and cancer cells (F.-C. Lin & Young, 2013), but can also downregulate lymphocyte migration (Raphael et al., 2015), as well as modulate the production of pro-inflammatory cytokines, induce apoptosis, and recruit anti-inflammatory mediators (e.g., IL-1 receptor antagonist, IL-18 binding protein; Mühl & Pfeilschifter, 2003).

One prominent compound that can be administered or measured in animals and humans to study a systemic immune response and related physiological outcomes is lipopolysaccharide (LPS) levels (or endotoxin; Andreasen et al., 2008). In most Gram-negative bacteria, LPS molecules are glycolipids that are present in the outer cell membrane and function as structural and barrier support for the bacteria (Bertani & Ruiz, 2018). In humans and animals, LPS is a type of pathogen-associated molecular pattern that is recognized as pathogenic by host immune cells that contain TCRs (Andreasen et al., 2008). This recognition results in various intracellular signalling pathways to activate the transcription factor, nuclear factor κB, and release pro-inflammatory cytokines (e.g., TNF-α) and other immune molecules to fight the pathogen (e.g., that help with cell migration, induce fever; Andreasen et al., 2008). This immune response does not act in isolation but is a sophisticated interaction between all bodily systems. For instance, the pro-inflammatory cytokines that are initially released in response to pathogen recognition can stimulate the release of other cytokines (e.g., IL-6) that work to suppress the immune response by various mechanisms (e.g., production of anti-inflammatory cytokines like IL-10, inhibition of pro-inflammatory cytokines like TNF-α; Andreasen et al., 2008).

1.4.3 Immune System Function in Situations of Stress

Almost a century ago, Selye (1936) performed experiments with rats that showed that various physiological stressors (e.g., excessive exercise, exposure to cold) resulted in immune, metabolic, and stress-related changes (e.g., decreased thymus size, reduction of fat tissue, enlargement of the adrenal glands). Today, it is recognized that the HPA axis directly interacts with the immune system; cytokines both directly impact the negative feedback of glucocorticoid release from the adrenal glands but also act on the hypothalamus (e.g., to affect CRF and AVP release) and the anterior pituitary (e.g., to

affect ACTH release; Chrousos & Gold, 1992; Karrow, 2006). As part of the innate immune system's localized inflammatory response to foreign invaders, both the HPA axis and autonomic nervous system are activated and impact inflammatory processes (Karrow, 2006). For instance, pro-inflammatory cytokines TNF-α, IL-6, and INF-γ have been shown to inhibit CRF-mediated ACTH release from rat pituitary cells (Vankelecom et al., 1990). As reviewed by van der Velden (1998), on the anti-inflammatory effects of glucocorticoids for the treatment of asthma, the administration of glucocorticoids has been shown to reduce protein levels of various pro-inflammatory and regulatory cytokines (e.g., IL-6, TNF-α, GM-CSF). Indeed, male Sprague–Dawley rats exposed to maternal separation stress show increased IL-6 (measured from whole blood culture supernatants) and CRF mRNA in the amygdaloid cortex, compared to non-separated rats (Desbonnet et al., 2010).

In adult male mice (BALB/c), exposure to psychological (communication box paradigm) or physical stress (foot-shock stress) in adolescence resulted in poorer adult asthma symptom profiles during an ovalbumin airway challenge (to induce asthma symptoms; Chida et al., 2007). Interestingly, the psychological stress exposure prevented the increased serum CORT that resulted from the airway challenge in both the control and physical stress groups (Chida et al., 2007). In a study with male Sprague–Dawley rats that were exposed to a maternal separation stress paradigm for 12 days post-birth, acute stress exposure (novel OFT) in early adulthood resulted in increased CORT and TNF-α after behavioural testing, compared to rats not exposed to the early life stressor (S. M. O'Mahony et al., 2009). Furthermore, a recent study with members of this prior research group examined the effects of maternal separation stress with concurrent immune stress (LPS) in female Sprague–Dawley rats (Nicolas et al., 2022). The authors found that, regardless of prior maternal separation stress, LPS resulted in increased plasma CORT and inflammatory cytokines, measured two hours after LPS injection (intraperitoneal) compared to saline injection. Notably, in the hippocampus, gene expression of IL-1β at 24 hours was increased with LPS injection in the dorsal hippocampus, regardless of prior stress exposure, but IL-1β expression was only increased in the ventral hippocampus if the female rats were previously exposed to maternal separation stress (Nicolas et al., 2022).

As complemented by these presented rodent studies, it is well-established that various types of cytokines (e.g., pro-inflammatory, anti-inflammatory, immunoregulatory) are produced during stress in humans (e.g., psychological stress in Kang & Fox, 2001; Maes et al., 1998; strenuous physical exercise in Northoff & Berg, 1991). Indeed, Carpenter et al. (2010) reported an association between childhood maltreatment (a form of early life stress) and elevated IL-6 production in response to laboratory psychosocial stress relative to a control group. In addition, higher self-reported psychological stress before infection with influenza A has been associated with more respiratory symptoms from the infection and higher IL-6 production (measured from nasal secretions; S. Cohen et al., 1999). Immune-related markers have also been shown to be altered generationally, whereby adult women from mothers who experienced negative life events during pregnancy had elevated levels of cytokines like IL-6 and IL-10 (Entringer et al., 2008). As well, increased pro-inflammatory markers have been reported in people with anxiety disorders and trauma- and stress-related disorders (e.g., IL-1β, TNF-α; Michopoulos et al., 2017; Passos et al., 2015).

1.4.4 Immune Responding and Metabolic Functioning

It is widely accepted that diet affects various aspects of health through interactions between host physiological responses and the microbiota. For instance, strict dietary changes (e.g., the autoimmune protocol diet) can be used to treat certain autoimmune diseases, sometimes in the absence of pharmacological therapy (e.g., irritable bowel disease; Konijeti et al., 2017; Hashimoto's thyroiditis; Abbott et al., 2019). The autoimmune protocol diet eliminates and reintroduces various foods thought to induce inflammation in people with autoimmune diseases (e.g., grains, dairy, coffee) in combination with lifestyle changes (e.g., improving sleep quality, reducing stress, increasing physical activity; Konijeti et al., 2017). However, changing diet and lifestyle factors is not always beneficial in all people and with all autoimmune diseases. While these changes can be incorporated into treatment plans (e.g., fish oil supplementation with rheumatoid arthritis), they should not exclusively be preferred over pharmacological treatment for certain autoimmune diseases (Bullock et al., 2018). Indeed, genetic risk combined with environmental perturbations (e.g., pollutants, smoking, poor diet) seems to be fundamental in the development of

autoimmunity of various forms due to direct impacts on the functioning of immune cells (e.g., systemic lupus erythematosus, rheumatoid arthritis; Ellis et al., 2014; Harris-Tryon & Bel, 2020; Tobón et al., 2010).

Chronic stress can result in glucocorticoid resistance at a cellular level, meaning that GRs are not binding cortisol as well as they should, which can, in turn, affect inflammatory processes (Bauer & Teixeira, 2019). Namely, GRs that make it to the nucleus can inhibit nuclear factor κB and shut down pro-inflammatory processes, which can have pleiotropic effects on the development of psychological, endocrine-related, and immune-related disorders (Bekhbat & Neigh, 2018). Indeed, metabolic syndrome, obesity, and T2DM are linked to systemic inflammation through adipose tissue cytokine release (e.g., leptin, IL-6; reviewed by Ellulu et al., 2017). Early human work with a small sample of early adult men linked IFN-γ administration to increased serum IL-6, resting energy expenditure, and plasma ACTH and cortisol (de Metz et al., 1999). Further, leptin has been described as a pro-inflammatory molecule and might be directly implicated in autoimmune diseases like multiple sclerosis (Matarese et al., 2010) but also in inflammation-associated conditions that are not caused by autoimmunity (e.g., T2DM, cancer; La Cava, 2017). Interestingly, male C57BL/6 mice who are fed an HFD (60% kcal fat, 20% kcal carbohydrate) for six months starting in adolescence have systemic inflammation and insulin resistance that persists even after they are switched to an SD (14% kcal fat, 54% kcal carbohydrate) that induces weight and fat loss (Blaszczak et al., 2020). This concept of an 'obesogenic memory' related to inflammation has been previously shown to be specific to adipose tissue in both previously HFD-induced obese (60% kcal fat; 20% kcal carbohydrate) male C57BL/6 mice and humans with obesity (Schmitz et al., 2016). In both groups, weight loss and reduced liver inflammation were observed (i.e., from a calorie-restricted diet in mice, bariatric surgery in humans), but adipose tissue inflammation persisted even with weight loss (Schmitz et al., 2016).

1.4.5 The Gut Microbiota-Immune Axis

It has become increasingly acknowledged that proper immune system development and function rely on the gut microbiota. Early in life, the gut microbiota and the immune system

homeostatically co-exist and work to maintain a healthy balance in the face of environmental factors. Importantly, the microbiota in the gut and elsewhere in the body (e.g., skin, lungs) help to shape host immunity during development, which has implications for the development of healthy and diseased states in the host (reviewed by Palm et al., 2015; Tomkovich & Jobin, 2016). Specifically, the gut microbiota is essential for immune system development (e.g., Th2-mediated immune responses) and is critical for suppressing immune responses to components in food and other innocuous antigens (Sudo et al., 1997). However, in some cases, the environment puts too much strain on the healthy cooperation between the immune system and the gut microbiota, which can result in altered immune responding and changes in healthy physiological functioning (e.g., antibiotics to pre-term infants; Greenwood et al., 2014; early life stress in male Sprague–Dawley rat pups; S. M. O'Mahony et al., 2009). Overall, both the microbiota and immune system develop from early life exposures (e.g., exposure to the birth canal, maternal milk consumption), and an appropriate (homeostatic) balance between these two systems has implications for lifelong health status (Belkaid & Hand, 2014).

The ability of the immune system to recognize, categorize, and defend appropriately to host cells compared to pathogens is already understandably complex; however, when also considering that the gut microbiota is comprised of non-host cells that can be beneficial or pathogenic, it is not unexpected that the immune system can become dysregulated, especially in response to genetic risk factors and harmful environmental stressors. As previously described, the immune system can be broken down into innate and adaptive responses, and the development and functioning of the immune system is influenced by the microbiota (Belkaid & Hand, 2014). It has been suggested that the evolution of an adaptive immune system was directly influenced by gut microbes (i.e., from both beneficial symbionts called commensals and from detrimental pathogens; Y. K. Lee & Mazmanian, 2010). Indeed, on top of the aforementioned behavioural and metabolic functioning deficits that occur with germ-free animal models, the immune system of germ-free mice does not develop appropriately without the presence of microbes in the host (Ericsson et al., 2021). As well, changes in commensal microbe presence in different strains of mice can vary drastically and be associated with alterations in anxiety-related behaviours (e.g., locomotor activity, time spent in center in the OFT, time spent in light in the LDB) and physiology

(e.g., body size, cardiac function, glucose tolerance, white blood cell numbers), sometimes differentially in both sexes (Ericsson et al., 2021).

The division of the immune system that functions in the gut (i.e., the gut mucosal immune system, or gut-associated lymphoid tissue) is a large component of a host's immune system with both innate and adaptive functioning (Holmgren & Czerkinsky, 2005). This division of the immune system is comprised of a gut mucosal membrane (i.e., endothelial tissue, lamina propria, muscularis mucosa) that consists of a variety of immune cells (e.g., immature lymphocytes of the adaptive immune system, macrophages of the innate immune system; Holmgren & Czerkinsky, 2005). Specifically, endothelial tissue in the gut contains specialized cells known as enterocytes and goblet cells that absorb substances from the intestinal lumen to the blood and produce mucus to protect from pathogens, respectively (García-Montero et al., 2021). Microbes and their metabolites are key for the development of the gut mucosal immune system (e.g., Peyer's patches, cells of the gut lining; Fiebiger et al., 2016). Changes in the functioning of the gut mucosal immune system have been directly linked to dysbiosis of the gut microbiota in this gut mucosa region, which can have implications for disease development (e.g., autoimmune diseases, cancer; Aghamajidi & Maleki Vareki, 2022; Kuhn et al., 2014). When it comes to the relationship between the gut microbiota and brain function, altered cytokine release is proposed to be one method of communication that can be disrupted in disease states (Shoubridge et al., 2022).

With the idea that the development of a functional and healthy immune system relies on the gut microbiota, it is foreseeable that autoimmunity can result when the gut microbiota is in a state of dysbiosis or not functioning optimally (Kuhn et al., 2014). As such, increased presence of LPS, which can result in systemic inflammation, can be measured to infer a dysbiotic state because increased LPS is characteristic of increased presence of pathogenic bacteria that harbour LPS (García-Montero et al., 2021). In a normal or healthy state, the fundamental roles of the immune system are to protect and defend against pathogens and ignore cells from the host (i.e., self-tolerance; Harris-Tryon & Bel, 2020). With autoimmune diseases, the proper function of cells of the immune system is disrupted, and they begin to target host organs (e.g., the small intestine in Celiac disease) or the entire body (e.g., joints, kidney, heart in systemic lupus erythematosus; Marrack et al., 2001).

Of course, a complete understanding of the mechanisms for autoimmune disease development continues to be elucidated, and a dysfunctional gut microbiota is only one factor that increases this risk (Kuhn et al., 2014). Indeed, it has been proposed that, in an individual with a genetic risk of an autoimmune disease, dietary components can influence the gut microbiota and alter immune responding to environmental factors that might lead to an autoimmune state (Vieira et al., 2014). The intestines function as a buffer between the body and environmental components (Rohr et al., 2020). The intestines also harbour the mucosal immune system that can protect hosts from pathogens and disease risk factors, so the integrity and proper function of this region are critical for health (Rohr et al., 2020). One key example of compromised barrier integrity resulting in disease is Celiac disease, where hyperpermeability to gluten results in inflammation in the small intestine at the level of the mucosal immune system leading to damage in the region and associated symptoms (e.g., pain, mucous formation; Stamnaes et al., 2021).

1.4.6 Effects of Diet on Immune Function

The gut microbiota is affected by dietary choices (Kau et al., 2011; Zmora et al., 2019), and this relationship can also influence the development of autoimmune diseases (Vieira et al., 2014). For example, in male Wistar rats, WD (45% kcal fat, 35% kcal carbohydrate) administration for 16 weeks resulted in increased expression of TNF-α, IL-1β, and IL-6 in the hypothalamus, compared to an HCD control (10% kcal fat, 70% kcal carbohydrate; De Souza et al., 2005). Similarly, with male Sprague–Dawley rats, Dutheil et al. (2016) report increased cytokine protein levels of IL-6 and TNF-α, along with increased IL-1β and IL-6 mRNA in hippocampal tissue of HFD-fed rats (16 weeks; 60% kcal fat, 20% kcal carbohydrate) compared to HCD control-fed rats (10% kcal fat, 70% kcal carbohydrate). Interestingly, HFD administration (60% kcal fat, 20% kcal carbohydrate) to male Sprague–Dawley rats, starting in adolescence, has also been shown to induce inflammatory cytokine increases (e.g., IL-1β protein in caecal lining), even if administered for only six weeks (compared to SD-fed rats; 18% kcal fat, 58% kcal carbohydrate; Crawford et al., 2019). In another study with 12-week-old male C57BL/6J mice, bacterial LPS levels in plasma were shown to increase in response to HFD administration (four weeks; 72% kcal fat, < 1%

carbohydrate; Cani et al., 2007). In this work, administering LPS to HCD control mice (8% kcal fat, 72% kcal carbohydrate) also resulted in increased body weight and cytokine expression, like what was observed in the HFD-fed mice (e.g., IL-6, TNF-α mRNA in the liver; Cani et al., 2007). Further, LPS receptor(CD14)-deleted mice, who are insulin-sensitive, had a delayed metabolic response (e.g., delayed insulin resistance, body weight increase) when fed HFD (Cani et al., 2007).

Human studies that link diet to immune function alterations often include measures of mental health, brain function, and metabolic function. As also evidenced by the animal work presented, obesity in humans often presents with markers of low-levels systemic inflammation, and increased inflammatory activity has been directly linked to the development of obesity and T2DM (Hotamisligil et al., 1996; Ryan & Delzenne, 2016; Wellen & Hotamisligil, 2005). In response to the eight-week administration of a diet high in saturated fats, increased expression of cytokines (e.g., IL-6, IL-10) in adipose tissue has been reported in people with excess abdominal fat (van Dijk et al., 2009). Another study, which included lean adults and adults with obesity, administered a diet high in a saturated fatty acid (i.e., palmitic acid) for three weeks and reported increased plasma TNF-α from baseline (Kien et al., 2015). In contrast, a recent study that administered a healthy MD for one year to an elderly sample found that, compared to baseline, there were alterations in specific microbial taxa that were positively associated with improved cognition and negatively associated with inflammatory cytokines (e.g., IL-17; Ghosh et al., 2020).

1.4.7 Effects of Probiotics on Immune Function

In male Wistar rats, probiotic treatment with a 3-strain combination of *Lacticaseibacillus rhamnosus* R0011*, L. helveticus* R0052, and *B. longum* R0175 in response to infection with enterotoxigenic *E. coli* (i.e., *E. coli* infection that results in diarrheal illness; strain ATCC 11303) improved reduced serum cytokines levels (i.e., IL-1α, IL-6, TNF-α, IFN-γ), while increasing anti-inflammatory cytokines (i.e., IL-4, IL-10; Bisson et al., 2010). More broadly, reduced inflammatory markers have been reported with probiotic treatment in preclinical studies (e.g., 8-strain combination probiotic to adult male Syrian hamsters in Avolio et al., 2019; 3-strain combination probiotic to adult male C57BL/6 mice in N. Li et

al., 2018; *Limosilactobacillus reuteri* 23272 to adult male CD1 mice in Mackos et al., 2013). Relatedly, *Companilactobacillus farciminis* treatment for two weeks (1×10^{11} CFU/day) to adult female Wistar rats mitigated increased LPS and hypothalamic cytokine mRNA expression (i.e., IL-1β, IL-6, TNF-α) induced by partial restraint stress, compared to a saline placebo (Ait-Belgnaoui et al., 2012). Moreover, in both male and female CD1 mice, one week of probiotic treatment with either Lacidofil® or CEREBIOME® (i.e., *L. helveticus* R0052 and *B. longum* R0175; 1×10^9 CFU/day), starting at five weeks of age, protected against LPS administration-induced cytokine increases, differentially in each sex (Esposito et al., 2022).

A recent randomized, double-blind, placebo-controlled trial in humans with asthma who were treated with a 7-strain combination probiotic (with *Lactobacillus* and *Bifidobacterium* strains, *Streptococcus thermophilus*, and a prebiotic) found that probiotic treatment reduced IL-4 in the probiotic group compared to pre-treatment baseline levels (Sadrifar et al., 2023). L. O'Mahony et al. (2005) demonstrated that administering *B. longum longum* 35624 to participants with IBS normalized the abnormal baseline IL-10/IL-12 ratios, along with improving symptoms related to their IBS (e.g., bloating, abdominal pain/discomfort). Gaisawat et al. (2022) exposed T84 cells (i.e., a human colonic adenocarcinoma cell line) to *Clostridioides difficile*-infected fecal water and found that treatment with various probiotic strains (e.g., *Saccharomyces boulardii* CNCM I-1079, *Lacticaseibacillus rhamnosus* R0011, *L. helveticus* R0052, *B. longum* R0175) reduced infection-associated increases in various pro-inflammatory cytokines. Finally, a randomized controlled trial by Lew et al. (2019) administered *Lactiplantibacillus plantarum* P8 or placebo to participants for 12 weeks and reported that plasma levels of the inflammatory cytokine, IFN-γ, were significantly lower after 12 weeks of treatment compared to placebo participants.

1.5 Preface to Dissertation and Research Goals

1.5.1 Research on Western Diet D12079B

As introduced in Section 1.1.6, administering rodent WDs has the goal of inducing obesity so that various outcomes related to understanding the development, progression, and

potential treatments for obesity in humans can be studied. WD D12079B is a purified commercially available diet (from Research Diets, Inc.); D12079B is a butter- (i.e., anhydrous milk fat) and sucrose-based unhealthy diet with cholesterol, that additionally includes minor amounts of corn oil as a fat source, along with corn starch and maltodextrin as carbohydrates. This WD has been used to study obesity-related outcomes, such as diabetes or metabolic syndrome models (e.g., administered to male Sprague–Dawley and Zucker-Sprague–Dawley rats for four weeks in A. N. Wang et al., 2022; to male and female metabolic-related receptor knockout mice on C57BL/6J background in Garcia et al., 2014) and for more general diet-induced obesity studies (e.g., male and female C57BL/6J mice for four to eight weeks in Sulston et al., 2016; male C57BL/6J for about 400 days in Yashiro et al., 2019). For instance, relative to a purified HCD control (10% kcal fat, 73% kcal carbohydrate), D12079B has been shown to increase body weight in middle-aged male C57BL/6 mice fed for 21 weeks, but the WD did not affect cytokine levels (IL-6, TNF-α, MCP-1) or cognitive tests (T-maze) compared to this control (Pistell et al., 2010). As well, with four weeks of administration to male and female C57BL/6J mice, WD D12079B did not result in any metabolic changes in females, but males had increased fat mass and mitochondrial respiration (a marker of obesity; McGowan et al., 2022).

One commonly used purified control diet from Research Diets that has been used for D12079B is D14042701 (i.e., butter-based; 10% kcal fat, 73% kcal carbohydrate; J.-T. Hwang et al., 2021; McGowan et al., 2022; Myles, O'Leary, Smith, et al., 2020; Myles et al., 2023; Naudin et al., 2020). Specifically, the commonly used D14042701 is a control diet with no added sucrose or cholesterol, with corn starch and maltodextrin as the carbohydrate sources. Compared to WD D12079B, the D14042701 control diet has an equivalent amount of overall protein, along with identical amounts of casein, methionine, cellulose, and vitamin and mineral mixes. Although D14042701 usually includes a red dye source (i.e., red dye #40), our laboratory group orders this diet without red dye (cat. #D14042701N) due to reports of harmful health consequences related to this dye (e.g., see Kobylewski & Jacobson, 2012 for a review). As well, since the control diet is reduced in butter fat and includes a high amount of carbohydrates that are white, this control diet is white in colour compared to the yellow colour of D12079B; thus, the diets are easily distinguishable based on those colours, even when working under red light. Variations of

purified control diets for D12079B have also been reported related to specific study objectives. For instance, Pistell et al. (2010) administered Research Diets diet 98052602 (10% kcal fat, 73% kcal carbohydrate), which is sucrose-, cholesterol-, and maltodextrin-matched to WD D12079B, but with corn starch increased and milk fat completely replaced with corn oil. A recent study used 98121701 as a control diet, which is identical to 98052602 but without added cholesterol (Amens et al., 2023). Because it is sucrose-matched to WD D12079B, 98121701 has also been used as a high-sucrose diet (de Sousa et al., 2021). Some other options for purified control diets for WD D12079B are D13100302, which is also corn oil-based, but without any sucrose (used in Dijkstra et al., 2020), and D13100303, which is comparable to D13100302 but without added cholesterol.

Cereal-based SDs have been used as control diets as they contain a more generalizable amount of carbohydrates and fibre that have been shown to maintain adequate nutrition (Warden & Fisler, 2008). Research has shown that changing mice from a WD to an SD can reverse certain harmful metabolic consequences of the WD (e.g., normalized glucose levels, total cholesterol, insulin; Chiñas Merlin et al., 2022). Of course, the use of SD control diets can also have drawbacks because the other ingredients in the chow can vary greatly from purified HFDs or WDs (e.g., soy in SDs, sucrose in WDs; Warden & Fisler, 2008). Nonetheless, SDs that have been used as a control for D12079B include LabDiet® 5001 (corn- and soybean meal-based; 13% kcal fat, 58% kcal carbohydrate; Myles et al., 2023), LabDiet® 5008 (A. N. Wang et al., 2022), and an unspecified SD (Garcia et al., 2014). LabDiet® 5001 has also been used as a control for a commonly used lard-based HFD from Research Diets (D12492; Licholai et al., 2018; Sasaki et al., 2013, 2014). Furthermore, along with LabDiet® 5053, LabDiet® 5001 has been reported to be the most commonly used diet in various institutions for any study (i.e., each diet used by 14% of survey respondents; Tuck et al., 2020).

Differences in the composition of both experimental and control diets in studies can have important effects on research findings and might, in part, help to explain inconsistencies found in diet-induced obesity studies in both behavioural and physiological measures. Kosari et al. (2012) noted the issues with using different types of WDs or HFDs (e.g., rodent strain differences in susceptibility to the negative effects of the diets, age of rodents,

length of diet administration, diet composition). They gave male Long–Evans rats an SD (7% kcal fat; 59% kcal carbohydrate), WD (40% kcal fat, 43% carbohydrate), or very high-fat HFD (81% kcal fat, 6% kcal carbohydrate) for 12 weeks, and demonstrated that the WD increased body weight, calorie intake, blood pressure, and triglycerides compared to both the HFD and SD (Kosari et al., 2012). Lang et al. (2019) gave male C57BL/6J mice an SD, a purified control diet with reduced carbohydrates and added fibre (13% kcal fat; 67% kcal carbohydrate), a WD (45% kcal fat; 35% kcal carbohydrate), or cafeteria diet (i.e., SD with salty and sweet snacks) for 12 weeks starting at six weeks of age. They found similar significantly higher weight gain, reduced glucose tolerance, and insulin resistance in both the cafeteria and HFD groups, compared to the SD or control diet groups (Lang et al., 2019). Interestingly, HCD controls and HFDs seem to be similar for weight gain when diet administration is short-term but begin to differentiate after longer administration (e.g., two months or greater; Dutheil et al., 2016; González et al., 2023).

1.5.2 Lactobacilli and Bifidobacteria

Lactobacilli is the trivial name for the genus *Lactobacillus*, which is part of the phylum Firmicutes, class Bacilli, order Lactobacillales, and family Lactobacillaceae (Widyastuti et al., 2021; Zheng et al., 2020). Before March 2020, the *Lactobacillus* genus contained a diverse 261 species of bacteria, but this genus was divided into 25 distinct genera (e.g., *Lactobacillus*, *Lacticaseibacillus*) due to shared functional and genetic properties (Zheng et al., 2020). Currently, taxa of the *Lactobacillus* genus (38 species, eight subspecies) are described as rod-shaped Gram-positive bacteria that primarily produce lactic acid, grow optimally at temperatures above human body temperature, and are acid-tolerant (e.g., *L. helveticus* grows best between 42 to 45 °C and at a pH between 5.5 and 5.8; Slattery et al., 2010; Zheng et al., 2020). More broadly, lactobacilli have been the focus of probiotic research due to their non-pathogenic nature and ability to bind to epithelial cells in the intestine (Reid & Burton, 2002). One general example of a probiotic effect of lactobacilli is the finding of reduced visceral pain in response to colorectal distension in male Sprague–Dawley rats who are given *Limosilactobacillus reuteri* 23272 (Kamiya et al., 2006). Clinically, the administration of strains of lactobacilli (i.e., *Lacticaseibacillus rhamnosus*

GMNL-74, *L. acidophilus* GMNL-185) has been shown to be antimicrobial against the opportunistic pathogen *Helicobacter pylori* (i.e., they affected the adhesion, function, and immune response to the pathogen), while also increasing the abundance of beneficial bacteria, such as *Bifidobacterium* species and *A. muciniphila* (Y.-H. Chen et al., 2019).

L. helveticus was first isolated from Emmental cheese, and this species produces lactic acid from specific sugar sources (i.e., glucose, galactose, lactose, mannose, trehalose; Zheng et al., 2020). *L. helveticus* strains make for effective probiotics due to their ability to survive and adhere in the gut, along with their ability to compete with gut pathogens, affect host immune response, and alter the composition of the gut microbiota (Taverniti & Guglielmetti, 2012). Although *L. helveticus* and *L. acidophilus* are closely related genetically, they are usually isolated from different places (i.e., *L. acidophilus* from animal digestive tracts, *L. helveticus* from fermented foods like dairy products; Taverniti & Guglielmetti, 2012). Due to this genetic closeness, CEREBIOME® (or Probio'Stick® as it was so previously named) has been reported to contain *L. acidophilus* Rosell-52 and *B. longum* Rosell-175 in research (e.g., Diop et al., 2008) because R0052 phenotypically resembles *L. acidophilus* (Foster et al., 2011). However, strain R0052 was isolated in 1990 from dairy milk cultures and most closely resembles *L. helveticus* genetically (Naser et al., 2006); it is referred to as a strain of *L. helveticus* today.

As reviewed by Foster et al. (2011), *L. helveticus* R0052 has been demonstrated in research to adhere to human intestinal epithelial cells (Sherman et al., 2005), affect immune system functioning *in vitro* (Easo et al., 2002; T. D. Wallace et al., 2003), and have antagonistic activity against *E. coli* (Atassi et al., 2006; Jandu et al., 2009; Johnson-Henry et al., 2007; Sherman et al., 2005), *Salmonella typhimurium* (Atassi et al., 2006), *Campylobacter jejuni* (Alemka et al., 2010; Wine et al., 2009), and *Staphylococcus aureus* (Sadowska et al., 2010). As a probiotic, *L. helveticus* strain R0052 has been described as transient, meaning it does not colonize the gut; it is either excreted with stool or destroyed during its passage through the gut (Firmesse et al., 2008; Foster et al., 2011). Studies that have tested for probiotic presence in the colonic mucosa (from colonic biopsies) suggest that some strains of administered bacteria can attach to gut mucosa and persist in this region for longer than in the feces (e.g., *Lacticaseibacillus rhamnosus* GG; Alander et al., 1999); however,

probiotic strain presence in gut mucosa also seems to be temporary, dependent on continued administration, and might even be genus-, species-, or strain-specific. For instance, in a BALB/c mouse model, *Lacticaseibacillus rhamnosus* Lcr 35 (1×10^9 CFU/day) was found to be recoverable in feces for about three days after stopping administration, in decreasing amounts each day (De Champs et al., 2003). As speculated by Alander et al. (1999), levels of administered probiotic strains will start to dwindle if administration stops due to competition from endogenous strains, which makes permanent colonization of any probiotic strain unlikely.

Bifidobacteria is the colloquial name for members of the genus *Bifidobacterium*, from the phylum Actinobacteria, class Actinomycetia, order Bifidobacteriales, and family Bifidobacteriaceae (J.-H. Lee & O'Sullivan, 2010). Bifidobacteria are non-pathogenic Gram-positive Y-shaped bacteria that are bile- and acid-tolerant (Bottacini et al., 2017; Westermann et al., 2016), grow slightly above normal human body temperature at a pH of 6.5 to 7.0 (Shah, 2011), and were first isolated from the feces of breast-fed infants (Tissier, 1899). Bacteria of the genus *Bifidobacterium* (51 species, ten subspecies; Bottacini et al., 2017), including *B. longum* strains, are commensally present in both the human gastrointestinal tract and vagina, and are essential for health-promoting bacterial diversity in these areas (Chaplin et al., 2015; M. A. Schell et al., 2002; C. Zhang et al., 2019). In the human gastrointestinal tract, these non-pathogenic bifidobacteria are present in high abundance early in life (Turroni et al., 2012) and seem to help hosts with physiological functions, such as digestion of non-digestible carbohydrates and production of B vitamins (reviewed by Rivière et al., 2016). The administration of strains of bifidobacteria has been repeatedly demonstrated to yield health benefits (e.g., treatment of infectious diarrhea, lactose intolerance, bacterial infections, constipation; reviewed by Leahy et al., 2005), although the molecular mechanisms for these effects are still being unravelled (Bottacini et al., 2014). Nevertheless, specific strains of bifidobacteria have been established as psychobiotics (see Section 1.1.7) due to their effects on reducing psychological symptomatology in both animal and human studies (e.g., *B. longum* 1714 to human participants in Allen et al., 2016; *B. longum infantis* CCFM687 to mice in Tian et al., 2019).

B. longum R0175 is less characterized in the literature compared to *L. helveticus* R0052, although Messaoudi, Lalonde, et al. (2011) confirmed its identity as a strain of *B. longum* (by 16S rRNA and *tuf* gene sequencing). At the species level, *B. longum* has been shown to adhere to human fecal mucus (F. He et al., 2001), which has been suggested to be important, albeit not necessarily critical, for a probiotic to provide a benefit to the host; although there does seem to be strain-specific differences in adhesion capacity with *B. longum* strains (E. Izquierdo et al., 2008). Furthermore, *B. longum* R0175 is not often studied and administered on its own (i.e., without concurrent R0052 administration), although limited studies do exist. For instance, in a rat model (male Sprague–Dawley) of acute liver failure with D-galactosamine, *B. longum* R0175 reduced the severity of liver injury, reduced various levels of plasma pro-inflammatory cytokines (e.g., TNF-α), and shifted the gut microbiota composition to a more beneficial state (K. Wang et al., 2020). In pigs, *B. longum* R0175 (identified as strain 75119 in the publication) administration has been shown to increase the abundance of bifidobacteria and reduce Clostridia (Estrada et al., 2001). Finally, a randomized, placebo-controlled study with participants with irritable bowel syndrome (IBS) that evaluated *Lacticaseibacillus paracasei* HA-196, *B. longum* R0175, or placebo, found that both probiotic strains on their own had beneficial effects on psychological functioning measures but that only *Lacticaseibacillus paracasei* HA-196 aided with symptoms of IBS (Lewis et al., 2020).

1.5.3 Research on CEREBIOME®

CEREBIOME® (previously commercially known as Probio'Stick®) is comprised of a balance of 90% *L. helveticus* R0052 (or Rosell-52, CNCM I-1722, K300, K1) and 10% *B. longum* R0175 (or Rosell-175, CNCM I-3470). Animal work on CEREBIOME®, as it relates to anxiety-related behaviours, stress, and more broad health-related outcomes, has been fairly extensive in the past decade or so; however, animal research using both sexes is scarce. In adult male C57BL/6 mice, this probiotic strain combination (1×10^9 CFU/day) has been linked to improvements in gut barrier permeability, reduced HPA axis activity (i.e., reductions in plasma CORT, adrenaline, noradrenaline), and reduced cFos following chronic stress (Ait-Belgnaoui et al., 2014). Also with adult male C57BL/6J mice,

CEREBIOME® treatment (1×10^9 CFU/day) decreased the HPA axis activation in response to chronic water avoidance stress (i.e., the probiotic reduced the increased plasma CORT and increased the reduced GR mRNA in the hypothalamus, hippocampus, and prefrontal cortex; Ait-Belgnaoui et al., 2018). With male Wistar rats, Messaoudi, Lalonde, et al. (2011) reported that two weeks of CEREBIOME® treatment (1×10^9 CFU/day) resulted in lower observed anxiety-related behaviours, compared to placebo rats, in the defensive burying test. In adult male Syrian hamsters, CEREBIOME® treatment at low and high doses altered microbial composition prior to stress exposure but had conflicting effects on anxiety-related behaviours depending on the dose (i.e., low dose of 1×10^9 CFU/day increased social behaviour deficits after acute social defeat stress, but high dose of 1×10^{10} CFU/day did not differ from placebo in this measure; Partrick et al., 2021).

More specific mechanistic animal studies that have administered CEREBIOME® have shown physiological and immune-related improvements in health. In an early study, CEREBIOME® treatment (1×10^9 CFU/day) reduced apoptotic-associated genes (e.g., caspase-3) in the amygdala and dentate gyrus (but not in the CA1 or CA3 regions) in adult male Sprague–Dawley rat after induction of a myocardial infarction (Girard et al., 2009), while also improving myocardial infarction-related depressive symptoms and normalizing the increased intestinal permeability that occurred after the myocardial infarction (Arseneault-Bréard et al., 2012). Furthermore, CEREBIOME® administration to male Wistar rats (1×10^9 CFU/day), who were also administered LPS (from *E. coli* 055:B5; to induce an inflammatory response), has been reported to decrease pro-inflammatory cytokines (i.e., TNF-α and IL-1β protein) in plasma and hippocampus (Mohammadi, Dargahi, Peymani, et al., 2019) and reduce markers of hippocampal apoptosis (e.g., BAX/BCL-2 protein ratio; Mohammadi, Dargahi, Naserpour, et al., 2019), compared to rats given a maltodextrin placebo. CEREBIOME® administration (1×10^{10} CFU/day) to male rats has also been reported to help mitigate the negative effects on liver function after a methyl-deficient diet (Sprague–Dawley rats; Tillmann et al., 2021) and reduce plasma dopamine that was elevated with depression (in Flinders Sensitive Line rats, a model of depression; Tillmann et al., 2018). Overall, these animal studies highlight the beneficial effects of CEREBIOME® treatment (often at 1×10^9 CFU/day) on both behavioural (e.g., reduced anxiety-related behaviours, reduced HPA axis response) and physiological

outcomes (e.g., restoration of gut barrier integrity, decreased pro-inflammatory cytokine release, reduced apoptotic response).

In humans, this combination of *L. helveticus* R0052 and *B. longum* R0175 (CEREBIOME®) is approved in Canada (NPN 80021343) as a natural and non-prescription health product for helping to promote broad psychological well-being (e.g., improve mood balance, symptoms of anxiety) and related gastrointestinal symptoms. One early study gave human participants (male and female; double-blind, placebo-controlled) that had symptoms of stress (e.g., nervousness, gastrointestinal disturbances) CEREBIOME® (3×10^9 CFU/day for three weeks; Diop et al., 2008). Results showed that the probiotic treatment resulted in reduced stress-associated gastrointestinal concerns (i.e., abdominal pain and vomiting; Diop et al., 2008). In 2011, Messaoudi and colleagues published results of two clinical studies (randomized, double-blind, placebo-controlled) showing that CEREBIOME® treatment for 30 days (3×10^9 CFU/day) resulted in reduced reported psychological symptomatology (e.g., depressive symptoms, anxiety symptoms, anger-hostility) and reduced urinary-free cortisol from baseline compared to placebo (in all participants in Messaoudi, Lalonde, et al., 2011; specifically in a subset of participants with low baseline urinary-free cortisol in Messaoudi, Violle, et al., 2011).

More recently, a randomized, double-blind, placebo-controlled trial on participants with major depressive disorder found that CEREBIOME® supplementation (1×10^{10} CFU/day, eight weeks) decreased depression scores on the Beck Depression Inventory and kynurenine/tryptophan ratios (linked to depressive symptoms) from pre-treatment scores (Kazemi et al., 2019) and increased serum BDNF levels (Heidarzadeh-Rad et al., 2020), compared to prebiotic or placebo groups. Of note, another randomized, double-blind, placebo-controlled trial with CEREBIOME® (lower dose of 3×10^9 CFU/day for eight weeks) in people with at least moderate depressive symptoms did not find that the probiotic was helpful in treating symptoms of low mood or at affecting plasma biomarkers (e.g., cytokine proteins, vitamin D, BDNF protein; Romijn et al., 2017). Furthermore, another recently published randomized, double-blind, placebo-controlled trial found that four weeks of CEREBIOME® (3×10^9 CFU/day) administration did not improve various measures of psychological function (e.g., emotional regulation, anxiety) in healthy

participants (Morales-Torres et al., 2023). Although, the authors found that in subjects given the probiotic, 'health behaviours' (e.g., physical activity, interpersonal relations; distinct from measured 'risk behaviours' and 'uncertain behaviour') predicted improvements in specific dependent measures from baseline (i.e., they had lower difficulty with emotional regulation, decreased anxiety, increased mindfulness attitude; Morales-Torres et al., 2023). It may be that CEREBIOME® treatment for symptoms of psychological distress might not be beneficial in all groups of people, or effects could be dose-dependent. Indeed, it is of interest that a recent clinical pilot study (open-label) demonstrated that CEREBIOME® treatment at the same dose (i.e., 3×10^9 CFU/day) to treatment-naïve patients with major depressive disorder was helpful in improving psychological symptoms and sleep quality after eight weeks of treatment (C. J. K. Wallace & Milev, 2021). Taken altogether, these clinical studies suggest that CEREBIOME® is beneficial in improving psychological function in humans, but they highlight that participant characteristics and baseline health status might affect findings.

1.5.4 Studying Sex Differences

In research on human physiological and psychological outcomes, both sex (a biological category) and gender (a sociological construct that can be independent of sex) are distinct variables that interact to inform health research (Krieger, 2003). Although it is evident that the field of health research is working to become representative of all sexes and genders, when it comes to the female sex specifically, there is still a proportion of clinical studies that do not enroll females without good reason (reviewed by Geller et al., 2018). Of course, the consideration of participant gender (in addition to sex), along with the inclusion of ethnic and racial minorities in biomedical research, is instrumental for the advancement of knowledge of the determinants of health and disease for everyone (reviewed by Konkel, 2015; Nowatzki & Grant, 2011). For instance, Zucker and Prendergast (2020) report on an overwhelming number of pharmaceuticals that differ in pharmacokinetics in females compared to males (e.g., higher blood concentration in females at the same dose), and these pharmacokinetics were a significant predictor of adverse drug reactions in females. While studies such as this are critical in demonstrating that there are sex- and gender-specific

differences in healthcare outcomes, as is often found in similar studies, the terms 'woman and man' and 'female and male' are used interchangeably to refer to sex differences, which complicates interpretations because it is not possible to distinguish between sex and gender in the results. As such, there has been a push for increased knowledge on the difference between sex and gender for decades because the terms are often used interchangeably, even though they are different constructs that both inform health (Doyal, 2003; Gahagan et al., 2015; Garofalo & Garvin, 2020; Greaves & Ritz, 2022). With all of this said, because this thesis is focused on rodent research, there will be a focus on the disparity of sex-specific research studies in this section.

Certain human psychological disorders (e.g., PTSD) are more prevalent in females and, at least partly, this increased risk has been linked to altered functioning of the HPA axis (see Bangasser & Valentino, 2014 for a review). Despite it being known since the 1960s that, compared to males, female rats seem to have greater plasma CORT release and differences in CORT metabolism in response to acute stress (e.g., Kitay, 1961) or at baseline (e.g., Critchlow et al., 1963), studies have only recently been highlighting the importance of including both sexes in animal research. For instance, if previously directly exposed to maternal separation stress, male Wistar rats show increased plasma ACTH after novel EPM exposure compared to females (Wigger & Neumann, 1999). In addition, St-Cyr, Abuaish, Spinieli, et al. (2018) demonstrated that male and female C57BL/6 mice whose dams were exposed to predator odour stress during gestation show noteworthy behavioural differences to different tasks in adulthood. For instance, adult female offspring of predator odour-exposed dams have reduced activity (i.e., distance travelled) in the EPM after restraint stress compared to control dam female offspring, with no difference in males (St-Cyr, Abuaish, Spinieli, et al., 2018). In contrast, in the mouse defence test battery, predator odour dam males chased by a rat predator puppet stopped and oriented less towards the puppet (a measure of risk assessment) than control males, with no difference in females (St-Cyr, Abuaish, Spinieli, et al., 2018). Evidently, different types (e.g., predation, social) and duration or timing (e.g., prenatal, early life, acute, chronic) of stress exposure seem to result in sex-specific HPA axis responses.

In addition to these reported behavioural differences in response to acute and chronic

stressors, it has been known for quite some time that glucocorticoid binding in the brain is not equivalent in male and female rats. Turner and Weaver (1985) demonstrated *in vitro* that specific brain regions of adult male and female Sprague–Dawley rats do not bind CORT in the same way (e.g., the female hippocampus showed greater affinity for CORT; the male hypothalamus showed greater affinity). As well, removing the ovaries from female rats significantly increased CORT binding in the hypothalamus compared to females with ovaries, whereas removing the gonads in male rats did not alter binding (Turner & Weaver, 1985). These findings from Turner and Weaver (1985) highlight how gonadal-related hormone release can differentially affect the regulation of the stress response. More recently, St-Cyr and colleagues (2017) reported that compared to control females, female offspring whose dams were exposed to predator odour during gestation had increased amygdala GR gene expression at birth (no difference by adulthood), but the female offspring from predator odour-exposed dams did have increased levels of the Fkbp5 gene in the amygdala in adulthood (St-Cyr et al., 2017), which is a gene for a regulatory protein (co-chaperone) that reduces GR protein activity in the cytosol of cells (Zannas et al., 2016).

Behavioural and molecular differences between male and female rodents in response to stress and during behavioural testing can be extended to metabolic and inflammatory outcomes. Compared to male rats, female adolescent Sprague–Dawley rats have been reported to have increased expression of ghrelin receptor mRNA in the lateral hypothalamic area than males (López-Ferreras et al., 2017). Furthermore, antagonizing the ghrelin receptor in this region reduces measures of feeding and weight in female rats only (López-Ferreras et al., 2017). Interestingly, male and female C57BL/6 mouse offspring from dams who were exposed to predator odour stress during gestation had sex-specific responses with respect to body weight (St-Cyr, Abuaish, Welch, et al., 2018). With males, predator odour-exposed offspring weighed less and consumed less food than control males (no difference in females; St-Cyr, Abuaish, Welch, et al., 2018). With females, the offspring exposed to prenatal predator odour had a higher activity level over a measured 24-hour period (no difference in males; St-Cyr, Abuaish, Welch, et al., 2018). Human findings also show links between inflammatory responses and body weight. For instance, postmenopausal women on hormone replacement therapy have reduced serum IL-6

compared to women not on hormone replacement therapy (Straub et al., 2000). Furthermore, an early review by Steinman et al. (2003) highlighted the conflicting findings that although leptin can promote autoimmunity, whereas research suggests CRF can be protective, females are at higher risk of autoimmune disease development but also produce more leptin and CRF than males.

1.5.5 Sex Differences with Diet and Probiotic Administration

Although much of the summarized research in Section 1.5.1 has been done exclusively in males, female rodents do not respond to diet-induced obesity studies in the same way as males. Thus, any focus on elucidating diet-specific changes in physiology and behaviour should also consider sex-specific outcomes. With both male and female rats, Gaur et al. (2022) recently compared the effects of an SD, SD supplemented with sucrose, or HFD (kcal% not reported) for ten weeks beginning in adolescence. They found that males were more metabolically susceptible to both the HFD and sucrose-supplemented SD than the females (Gaur et al., 2022). Sex-specific differences in response to cafeteria diet in rats have also been reported in Sprague–Dawley rats (e.g., impaired glucose tolerance in males, anxiolysis in females; Warneke et al., 2014). Maniam and Morris (2010) determined that cafeteria diet administration seemed to mitigate the anxiogenic effects of early life prolonged maternal separation in both adult male and female Sprague–Dawley rats, as compared to short-term maternal separation, which seemed to be beneficial in this respect and not affected by the cafeteria diet administration. In both sexes, rats exposed to both prolonged maternal separation and cafeteria diet (compared to short-term maternal separation and cafeteria diet) had increased hippocampal GR mRNA and normalized levels of hypothalamic CRF (Maniam & Morris, 2010). Interestingly, in the control group (non-handled), female rats displayed fewer anxiety-related and depressive-related behaviours than males and, in all groups, did not differ in plasma CORT levels (plasma CORT was reduced in males in the short-term maternal separation compared to the other two groups; Maniam & Morris, 2010). In another study with Long–Evans rats, female offspring exposed to HFD (60% kcal fat, 20% kcal carbohydrate) perinatally (i.e., four weeks prior to mating, during pregnancy, during lactation) had higher GR mRNA in their amygdala

compared to SD-fed (13% kcal fat; 58% kcal carbohydrate) females, with no difference in males from the diet (Sasaki et al., 2013).

Sex differences in the development of the gut microbiota have been reported to affect risk of disease (reviewed by Jašarević et al., 2016). For instance, J. He et al. (2019) reported that *Limosilactobacillus reuteri* DMSZ 8533 administration to BALB/c mice results in distinct changes to the gut microbiota (e.g., overall abundance of Bacteroidetes and Firmicutes phyla) in males compared to females. A recent study with male and female C57BL/6N mice who were fed HCD control (10% kcal fat, product number not reported) or WD (45% kcal fat, product number not reported) and treated with *Lacticaseibacillus rhamnosus* 0030 (1×10^8 CFU/day via oral gavage) or control (PBS), found sex-specific behavioural and metabolic changes (M. Schell et al., 2023). Specifically, prior to beginning probiotic treatment, while both males and females who were fed the WD for six weeks had increased body weight, females also had increased blood glucose and no change in insulin, whereas males had increased insulin and no change in blood glucose (M. Schell et al., 2023). When the probiotic (or control) was added, and diet administration continued for another six weeks, females fed the diet had increased leptin but no longer had increased insulin compared to HCD-fed females (M. Schell et al., 2023). In contrast, the probiotic did not seem to influence the deteriorating metabolic health (e.g., obesity, elevated leptin and insulin) of males in the study, but, in the males only, probiotic administration improved depressive-like behaviour (i.e., reduced immobility in the forced swim test compared to males given the control for the probiotic and WD; M. Schell et al., 2023).

1.5.6 Studying Anxiety and Obesity

Mice with diet-induced obesity (i.e., early adult male C57BL/6J mice fed 60% HFD) show poorer metabolic health and increased anxiety-related behaviours and, additionally, present with insulin resistance and neural inflammation in limbic structures (Soto et al., 2018). Interestingly, in this work, antibiotic treatment was shown to improve insulin responsiveness, reduce inflammation, and reduce anxiety-related behaviours, with concurrently associated changes in neurotransmitters, amino acids, and BDNF that could be induced in germ-free mice that received a fecal transplant (Soto et al., 2018). Indeed,

specific taxa of gut bacteria can directly contribute to the production and catabolism of neurotransmitters, such as monoamines (e.g., catecholamines like norepinephrine and dopamine; indolamines like serotonin) and amino acids like GABA and glutamate (reviewed by Y. Chen et al., 2021). For example, some species in the genus *Staphylococcus* produce trace amines from aromatic amino acids, with the enzyme staphylococcal aromatic amino acid decarboxylase, to produce dopamine and serotonin for the host through a biochemical pathway (Luqman et al., 2018). The drug metformin, a drug used to help with T2DM because it lowers blood glucose, has been shown to improve metabolic parameters in response to HFD feeding (60% kcal fat, 20% kcal carbohydrate), but also normalize anxiety-related behaviours that were increased from the HFD (early adult male C57BL/6J mice; compared to a 10% kcal fat, 70% kcal carbohydrate HCD control; Ji et al., 2019). In line with these metabolic and behavioural alterations, the HFD-fed mice given metformin had taxonomic differences compared to both the HFD-fed mice who were administered saline and compared to the HCD-fed mice (e.g., significantly reduced *Streptococcus* in HFD-metformin mice; Ji et al., 2019).

Human anxiety disorder prevalence rates can vary depending on the type of measure used to diagnose (e.g., PTSD included or not included, DSM vs. the International Classification of Diseases) and due to language and cultural differences, but globally, lifetime prevalence rates have been reported to be between 11.1% and 33.7% (reviewed by Bandelow & Michaelis, 2015). In 2006, a report was released on mental health in Canadians that stated that in 2002, about 1 in 8 Canadians between the ages of 15 and 64 reported symptoms that met the criteria of at least one anxiety disorder during their lifetimes (anxiety disorders would include PTSD and OCD at this time; Minister of Public Works and Government Services Canada, 2006). In Canada, obesity and overweight are prevalent conditions, with 26.8% of Canadian adults (18 years of age and up) being classified as 'obese' (i.e., BMI $\geq$ 30.0 kg/m^2) and 36.3% being classified as 'overweight' (i.e., BMI between 25.0 kg/m^2 and 29.9 kg/m^2; Statistics Canada, 2019). Globally, 39% of adults (18 years of age or older) have been classified as 'overweight' and 13% of adults as 'obese' (by BMI; World Health Organization, 2021). Although not exclusively, people classified as 'overweight' or 'obese' by BMI are at a higher risk of developing secondary health conditions (e.g., T2DM, heart disease; World Health Organization, 2021). For instance, in 2018, the prevalence of

T2DM was reported at 13.4% in people classified as 'obese', compared to 2.9% in people classified in the 'normal' BMI category (i.e., BMI between 18.5 kg/m^2 and 24.9 kg/m^2; Statistics Canada, 2019).

Anxiety and obesity are often comorbid health conditions that affect the risk of developing the other (Avila et al., 2015; De Hert et al., 2011; Rajan & Menon, 2017; Simon et al., 2006). Specifically, the symptom profiles and complications of each condition can overlap and affect the other (e.g., fatigue or self-esteem issues in obesity can worsen anxiety symptoms; de Wit et al., 2022). In fact, in a large population-based survey with about 175,000 participants, anxiety disorder prevalence has been reported to be significantly higher in both underweight (BMI < 18.5 kg/m^2) men and women and men in the 'obese' class III category (BMI ≥ 40.0 kg/m^2; G. Zhao et al., 2009). For women, anxiety disorder prevalence was also highest in the 'overweight' category and general 'obese' category (G. Zhao et al., 2009). In germ-free mice and rats with no microbiota, studies have reported protection from diet-induced obesity (Bäckhed et al., 2004, 2007) and reduced anxiety-related behaviours (Heijtz et al., 2011). However, these reported benefits exist alongside research that demonstrates increased susceptibility to diet-induced obesity (Fleissner et al., 2010), hyperactivation of the HPA axis (Sudo et al., 2004), disrupted responses of the immune system to immunization (Lamousé-Smith et al., 2011) and pathogens (Oliveira et al., 2005), and vitamin deficiencies (Sumi et al., 1977; Wostmann, 1981). Even with these conflicting findings, there does appear to be a connection between anxiety and obesity, that may depend, in part, on the gut microbiota. As such, anxiety and obesity are important to study together in the context of gut microbiota changes or interventions.

In general, higher prevalence rates of both obesity and anxiety disorders are reported in women compared to men (Baxter et al., 2013; GBD 2015 Obesity Collaborators, 2017). While the World Health Organization has reported that 2016 global estimates of obesity are higher in 'women' than 'men' (equal for the 'overweight' category; World Health Organization, 2021), 2018 Canadian prevalence rates of both 'overweight' and 'obese' categories are almost exclusively higher in 'males' than 'females' (Statistics Canada, 2019). One longitudinal study found that in middle-aged women, both the presence of metabolic syndrome and GAD were independently predictive of 10-year cardiovascular

mortality (Butnoriene et al., 2015). In addition, Jorm et al. (2003) reported that obesity in women, but not in men, was associated with significantly higher reported anxiety symptomatology (e.g., poor sleep, difficulty relaxing). In fact, one study in Iran found that even when accounting for sleep status, people with overweight or obesity reported more prevalence of anxiety and depressive disorders and that being a woman was associated with more anxiety (Sharafi et al., 2020).

Above and beyond reports that show differences in prevalence rates of anxiety and obesity by sex and gender, anxiety and obesity do not have the same clinical presentation in all sexes and genders. With obesity, females and males have differences in adipose tissue deposition (e.g., due to estrogen levels), which can affect the risk of and protection from secondary conditions such as cardiovascular disease (B. F. Palmer & Clegg, 2015). With anxiety, the psychosocial factor of degree of masculinity has been reported to be protective against the development of anxiety disorders because it presents with protective factors (e.g., self-confidence), highlighting the importance of considering gender distinctly from sex in human research (Farhane-Medina et al., 2022). Gender-based differences can also affect the presentation of comorbid conditions with anxiety disorders (e.g., women present most often with comorbid anxiety and depression; men present most often with comorbid anxiety and substance use problems; Farhane-Medina et al., 2022). When it comes to biological sex, a recent twin study found that methylation at the serotonin transporter gene and psychological symptomatology was increased in females and, specifically, methylation and somatization (i.e., the physical presentation of psychological symptomatology) were positively associated in females (Palma-Gudiel et al., 2019). Evidently, research that considers both gender and sex differences is critical if the goal is to inform the etiology and treatment of anxiety and obesity.

1.5.7 Objectives and Outline of Dissertation

The gut microbiota has been described as an endocrine organ (Busnelli et al., 2019; Clarke et al., 2014; Woźniak et al., 2021), as a metabolic organ (Guinane & Cotter, 2013; Rocha & Laranjinha, 2020; Stephens et al., 2018), and, more generally, as a microbial organ (Khoruts & Sadowsky, 2011; Turroni et al., 2020). Even though some reports do not agree

with distinguishing the gut microbiota as an organ as it could result in confusion and restriction about the multifaceted functionality of the microbiota (Riccio & Rossano, 2020), it is overwhelmingly evident that the gut microbiota is critical in affecting host risk of health and disease. Specifically, host enteroendocrine cells in the epithelium of the gut mucosa are crucial for responding to metabolites from the gut microbiota (e.g., by releasing metabolic hormones, cytokines, neurotransmitters), which, in turn, affects endocrine, metabolic, and immune function (Woźniak et al., 2021). As such, the gut microbiota, along with the associated genes in the gut microbiome and metabolites that are produced, can modulate physiological responses that affect animal host health or disease states.

As highlighted throughout this Chapter, the physiological systems impacted by the gut microbiota that affect endocrine, metabolic, and immune function are heavily intertwined and do not exist in isolation. The characterization of these systemic links is an ongoing and prolific area of research, but one example would be the fact that leptin and IL-6 function together to promote satiety, and both have been described as adipocytokines due to their overlapping physiological functions (Ahima, 2006; Cano et al., 2009; M. S. Han et al., 2020; Maachi et al., 2004). One function of cytokines like IL-6 is also to stimulate cortisol production, which binds to GRs (transcription factors; see Section 1.2.2) to further inhibit cytokine transcription (Andreasen et al., 2008). On the other hand, it has been shown that administering CORT to early adult male Sprague–Dawley rats two hours prior to an LPS immune challenge heightens the resulting cytokine expression (e.g., TNF-α mRNA in the liver and hippocampus), but CORT administration one hour after LPS generally results in reduced cytokine expression peripherally and centrally (i.e., an anti-inflammatory effect; Frank et al., 2010). Interestingly, for IL-6, the authors report that CORT administration after the LPS challenge did not reduce the LPS-induced increase in IL-6 hippocampal mRNA (i.e., opposite to what occurred in the liver and with other cytokines, e.g., TNF-α), and CORT administration prior to the LPS challenge significantly increased IL-6 mRNA compared to LPS only (Frank et al., 2010).

Research on the various models of unhealthy diet demonstrates that there are both behavioural and physiological changes in response to diet administration that differ by factors such as length of administration, type of control for the unhealthy diet, strain of

rodent or other animal, age of animal, sex of subjects, and additional experimental manipulations (e.g., stress exposure, treatment with drugs or other substances). For instance, diet has been shown to guide and change microbiota development in rodents (e.g., Crawford et al., 2019; Morrison et al., 2020) and humans (e.g., David et al., 2014; G. D. Wu et al., 2011). Furthermore, unhealthy diet administration has been shown to interact with different types of stress exposure (e.g., Sasaki et al., 2013) to affect the presentation of anxiety-related behaviours. Diet can also affect behaviour even without laboratory stress exposure (e.g., increased anxiety-related behaviours in Dutheil et al., 2016; reduced anxiety-related behaviours in Demir et al., 2022). In rodent studies, WDs and HFDs are also a robust factor in impairing metabolic (e.g., J.-H. Shin et al., 2018; Y. Yang et al., 2014) and inflammatory parameters (e.g., Cani et al., 2007; De Souza et al., 2005).

Throughout this Chapter, probiotics have also been demonstrated to affect the development and maintenance of the gut microbiota, which can have striking implications on health, depending on probiotic strain-specific factors and experimental decisions. For instance, *B. longum longum* 35624 is well established for its role in alleviating symptoms of irritable bowel syndrome in humans (Altmann et al., 2016; S. M. O'Mahony et al., 2009), whereas Lacidofil® is established as being a probiotic that improves stress coping parameters and associated gut abnormalities in rodents (Gareau et al., 2007, 2011; Zareie et al., 2006) and gastrointestinal function in humans (e.g., treatment of antibiotic-associated diarrhea; Song et al., 2010). As delineated in detail throughout this introduction, virtually all health outcomes are impacted by sex and gender, directly or indirectly. Even with sex and gender being key factors that affect health, they are unfortunately understudied and poorly designated in animal and health research.

As highlighted in Section 1.5.3, CEREBIOME® is commonly studied in mechanistic animal models and with human participants (by gender or sex), but animal work that includes female rodents is severely lacking. Although animal research with Western diet (D12079B specifically) more commonly studies female rodents, compared to the literature on CEREBIOME®, there is still a lack of studies with this diet that directly compare health-related outcomes in male and female rodents (some exceptions: McGowan et al., 2022; Myles, O'Leary, Smith, et al., 2020; Myles et al., 2023; M. G. Pitts et al., 2020). While

CEREBIOME® and other probiotic formulations commonly have a specific placebo comprised of excipients without microbial strains, research on Western diets is not as standardized; thus, findings are muddled with terminological differences (e.g., differences in what is considered an HFD, WD, or cafeteria diet). Furthermore, the type of control for diet-induced obesity models (e.g., various SDs, purified commercial control diets) is not standardized in the field, and discussions and debates on optimal control diets have been reported and are currently underway (e.g., González-Blázquez et al., 2020; Ricci, 2015; Warden & Fisler, 2008). Of course, while methodological differences cannot ever be fully controlled and specific experimental decisions are warranted depending on the goals and feasibility of studies, attempts to increase reporting on methodological differences in our own research and when discussing the research of others can aid with interpretations.

The work presented in this dissertation aims to increase current understanding of psychological and physiological changes in response to specific nutritional exposures to male and female Long–Evans rats. As will be presented in Chapter 2, the results in Study 1 provide insight into certain sex-specific anxiety- and metabolic-related outcomes in response to two types of commonly used control diets (i.e., a non-purified SD, a purified HCD control). Study 1 is a published manuscript in *Behavioural Brain Research* (Elsevier) whose use in the thesis is allowed due to author copyright permissions being maintained for reuse in dissertations. With both sexes of Long–Evans rat, this study administered a WD (Research Diets, cat. #D12079B; 40% kcal fat, 43% kcal carbohydrate) in direct comparison to one of its available purified HCD controls (Research Diets, cat. #D14042701; 10% kcal fat, 73% kcal carbohydrate) or SD (LabDiet® 5001; 13% kcal fat, 58% kcal carbohydrate). This work characterized specific anxiety-related behaviours and metabolic functioning outcomes in both sexes who were administered one of the three diets from weaning until early adulthood. Supplemental information for this manuscript is included at the end of this dissertation (Appendix A) as it appears in the published manuscript.

Study 2 (Chapter 3) presents work for a manuscript in preparation for submission that administered both the same WD as Study 1 (compared to the same SD) and CEREBIOME® (compared to excipient only placebo), again to both sexes of Long–Evans

rats. Supplemental information in preparation is also provided as supplementary tables and figures at the end of this dissertation (Appendix B). Study 2 parallels Study 1 in that anxiety-related and metabolic functioning outcomes were characterized in both sexes of Long–Evans rats, but in response to both WD and probiotic treatment, compared to control groups (i.e., placebo or SD). This study has the goal of expanding on findings from Myles, O'Leary, Smith, et al. (2020) that showed sex-specific anxiolytic effects of the probiotic, mitigation of worsening metabolic parameters with Western diet administration (compared to a purified HCD control), and changes in cytokine levels in plasma. Study 2 further adds to the understanding of the sex-related interactive effects of diet administration and probiotic treatment, which are nutritional factors that can affect the development and maintenance of the gut microbiota and health. In Chapter 4, results of both Study 1 and 2 will be placed into the context of the larger field of behavioural and molecular neuroscience. Collectively, this dissertation is presented to be applicable and accessible to an array of basic science and health-related fields, as certain central themes can be applied to research in multiple disciplines over and above the specific research findings of the two studies that will be presented.

CHAPTER 2 A COMPARATIVE STUDY OF WESTERN, HIGH-CARBOHYDRATE, AND STANDARD LAB DIET CONSUMPTION THROUGHOUT ADOLESCENCE ON METABOLIC AND ANXIETY-RELATED OUTCOMES IN YOUNG ADULT MALE AND FEMALE LONG–EVANS RATS

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Highlights

- Sixty Long–Evans rats were compared on anxiety-related and metabolic outcomes.
- Males given Western diet showed more stretch attend postures.
- Western diet and high-carbohydrate diet animals did not differ in weight gain.
- Western and high-carbohydrate diets increased plasma leptin in males only.
- At sacrifice, males were higher than females in hippocampal CA3 BDNF.

[†]The manuscript (format adapted for thesis; including a change from American to Canadian English and updated diet terminology in the discussion to be consistent with the other chapters) has been published as: Myles, E. M., Hamm, S. I., Allden, S. N., Romkey, I. D., O'Leary, M. E., & Perrot, T. S. (2022). A comparative study of Western, high-carbohydrate, and standard lab diet consumption throughout adolescence on metabolic and anxiety-related outcomes in young adult male and female Long–Evans rats. *Behavioural Brain Research*, 114184. https://doi.org/10.1016/j.bbr.2022.114184.

2.1 Abstract

Anxiety and obesity are prevalent health concerns that are affected by diet in rodents and humans. How diet influences the development and maintenance of anxiety and obesity has been challenging to characterize, in part, due to methodological differences in chosen experimental and control diets. Within the same experiment, anxiety- and obesity-related effects were characterized in rats fed a Western diet (WD) relative to two control diets. Sixty Long–Evans rats split equally by sex were given standard diet (SD), control (i.e., high-carbohydrate) diet (HCD), or WD from weaning until sacrifice in early adulthood. Anxiety-related behaviour was characterized in a modified open field test (mOFT) that allowed for the measurement of defensive behaviours (e.g., hiding within a refuge area), in addition to traditional OF measures (e.g., time in center). Both anxiety-related behaviours and hippocampal CA3 BDNF revealed specific sex differences. Neither adolescent weight gain of male and female rats, nor total body weight in early adulthood, were dependent on administration of HCD or WD, although the WD group consumed the most calories. In males only, administration of either WD or HCD resulted in elevated leptin levels relative to administration of the SD. Results indicate that SDs and HCDs are two distinct types of control diets that can affect comparability of studies and that using an SD might reveal more subtle metabolic changes. Control diet choice should be strongly considered during study design and interpretation, depending on specific research goals. Such studies should include both males and females as these effects are sex-specific.

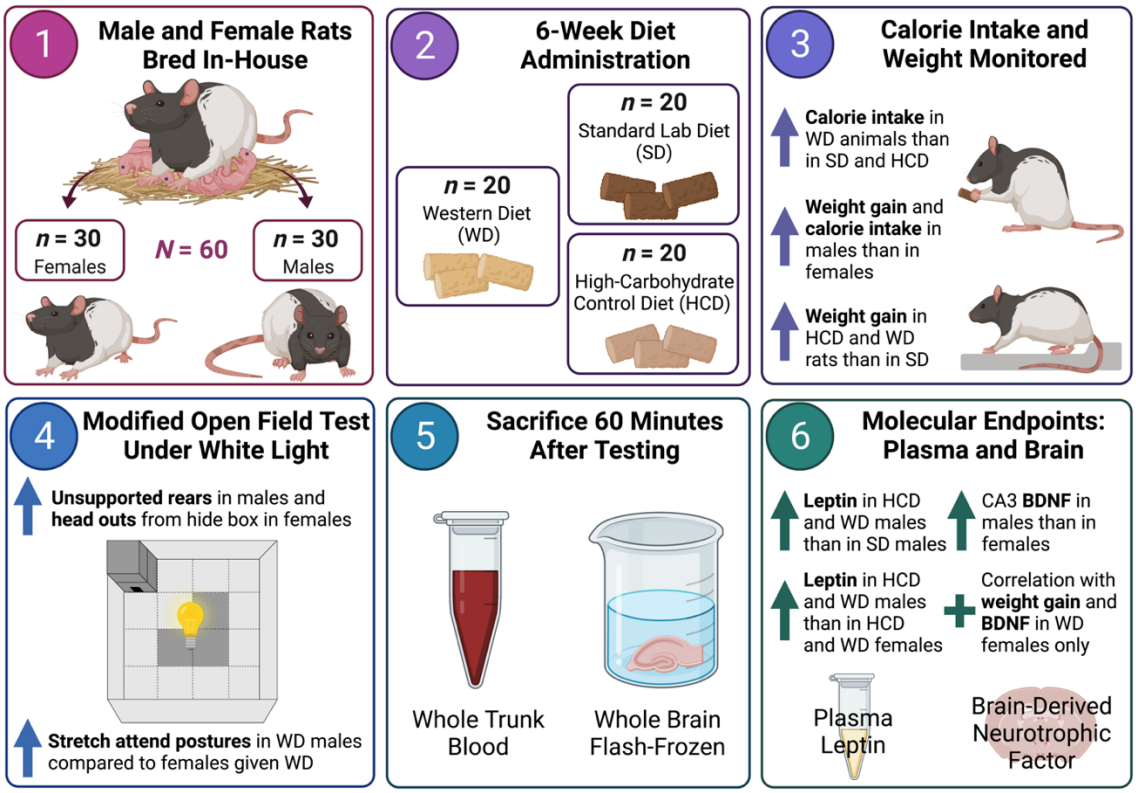


Figure 2.1 Graphical abstract depicting the research project, research timeline, and key findings.

2.2 INTRODUCTION

In 2015, it was estimated that 39% of the global adult (i.e., > 20 years old) population was 'overweight' or 'obese' (using the BMI scale; Chooi et al., 2019). Furthermore, prevalence rates of overweight and obesity are often found to be higher in women (e.g., from low-income areas, 50 – 65 years old) compared to men (Chooi et al., 2019). Obesity is a complex disease, but, in the most basic terms, it is due to incoming energy sources exceeding those that are internally expended and subsequently stored as adipose tissue in the body (Chooi et al., 2019). Although more difficult to determine, the global prevalence of anxiety disorders has been estimated to be around 7.3% (Baxter et al., 2013), and anxiety disorders are among the most prevalent mental health concerns (Gariepy et al., 2010; Stein et al., 2017). Anxiety disorders and obesity have consistently been shown to be interrelated (Baker et al., 2017; Deal et al., 2020; Gariepy et al., 2010). Indeed, people with depressive and anxiety disorders have poorer diet quality (Gibson-Smith et al., 2018), and improvements in symptoms are seen when interventions related to diet, exercise, and behaviour are introduced (Null & Pennesi, 2017). Further, unbalanced diets (e.g., comprised of processed foods, high in fat or sugar) are associated with an increased risk of psychological symptoms (e.g., concentration and sleep difficulties) often related to depression and anxiety (Jacka et al., 2010).

Nonetheless, characterizing the relationship between obesity and anxiety in terms of directionality and severity remains tenuous. Recently, it has been speculated that the hormone leptin, which is associated with Western diet (WD)-induced obesity (L.-L. Hwang et al., 2010; S. Lin et al., 2000; Lutz & Woods, 2012), may help explain the relationship between diet-induced obesity and anxiety (N. Yamada et al., 2011; Zemdegs et al., 2016). An imbalance in leptin could potentially Influence the development of mental health disorders, such as anxiety (Cernea et al., 2019). For instance, previous work suggests that serum leptin levels are elevated in humans who perceive greater psychological stress than in those who self-report lower levels of psychological stress (Otsuka et al., 2006). Furthermore, in patients with type 2 diabetes mellitus (T2DM), anxiety symptoms have been positively associated with serum leptin levels (Cernea et al., 2019). It has also been reported that rats administered a 20% sucrose solution with their regular standard chow for

24 weeks have elevated plasma leptin and engage in more anxiety-related behaviours (e.g., in the elevated plus maze [EPM], open field test [OFT]) than rats not given sucrose with their chow (Rebolledo-Solleiro et al., 2017).

One downstream target of leptin that could be involved in the mechanism mediating anxiety and obesity is brain-derived neurotrophic factor (BDNF). Reduced BDNF has been found in humans with anxiety disorders (Dell'Osso et al., 2009; I. M. dos Santos et al., 2011; Ströhle et al., 2010) and rats exposed to social isolation stress (review by Murínová et al., 2017). Specifically, reduced hippocampal BDNF has been reported in both *db*/*db* and diet-induced obesity C57BL/6J male mice (Stranahan et al., 2011; Yamada-Goto et al., 2012). Further, BDNF administration has been shown to ameliorate metabolic dysfunction in male rodents (Sprague–Dawley rats in ChuanFeng Wang et al., 2010; C57BL/KsJ-*db*/*db* mice in Nakagawa et al., 2000). In rats, the CA3 region of the hippocampus is relevant as this area has been reported to have the highest levels of BDNF mRNA in the rat central nervous system (Conner et al., 1997). Additionally, WD exposure in dams during gestation has been shown to impact the relationship between CA3 region leptin receptor expression and memory performance in offspring (Cortés-Álvarez et al., 2022). Furthermore, decreased BDNF mRNA has been previously reported with WD exposure in the CA3 and dentate gyrus regions of the hippocampus (Molteni et al., 2002). BDNF levels have also been reported to be reduced in mice exposed to poor diet (WD and no additional stress exposure in Molteni et al., 2002; 60% high-fat diet [HFD] and Forced Swim Test in N. Yamada et al., 2011). Further, leptin administration can increase levels of whole hippocampal BDNF in mice that are not fed an HFD (N. Yamada et al., 2011). Recent work in mice by Chen Li et al. (2021) has also demonstrated that leptin administration increases whole hippocampal BDNF mRNA, whereas leptin receptor deficiency decreases BDNF mRNA due to epigenetic modifications at *Bdnf* gene promotors.

Although there has been a concerted effort to study causal factors and mechanisms of anxiety- and obesity-related disorders to inform human outcomes, there is conflicting evidence as to whether a WD increases or decreases anxiety-related behaviours in rodents. For instance, studies have found that rodents fed a WD show increased anxiety-related behaviours in behavioural tests, such as the OFT, light-dark box (LDB), and the EPM (de

Noronha et al., 2017; Dutheil et al., 2016; Sivanathan et al., 2015). Yet, other studies have found that rodents fed a WD show decreased anxiety-related behaviours in the EPM, latency to step-down test, and the Barnes maze (Demir et al., 2022; Maniam et al., 2015; McNeilly et al., 2015; Ohland et al., 2016). Discrepancies in the results of past studies that examine anxiety-related behaviour changes in response to experimental manipulations (e.g., diet) may be due to several factors. For instance, various available tests for measuring such behaviour can show inconsistent results even when used in the same experiment (e.g., Korgan et al., 2018; T. P. O'Leary et al., 2013; Sturman et al., 2018). Furthermore, although commercial rodent diets designed to be comparable to the dietary composition of a human WD are used to study the development of anxiety (e.g., Hintze et al., 2018; Murphy & Mercer, 2013), the dietary composition (e.g., % fat, % carbohydrates) of HFDs, WDs, and the chosen control or standard chow diets can impact results and interpretations (Pellizzon & Ricci, 2018, 2020; Warden & Fisler, 2008). Many commercially available control diets are unavoidably high in carbohydrates, and research has shown that high-carbohydrate diets (HCDs) can have an adverse effect on anxiety-related behaviours (J. A. S. Gomes et al., 2020; C. J. Santos et al., 2018), reduce BDNF levels (Maioli et al., 2012), and increase leptin levels (Bursać et al., 2014; J. A. S. Gomes et al., 2020), similar to the adverse effects of WDs (Eudave et al., 2018; Kalyan-Masih et al., 2016; Kesby et al., 2015). Notably, recent work has begun providing a comparative analysis of how different types of laboratory diets affect specific metabolic parameters (e.g., Wistar rats of both sexes in Gaur et al., 2022; male C57BL/6J mice in Lang et al., 2019).

The objective of the present study was to increase available research on the effects of different rodent diets by characterizing both psychological and physiological outcomes of a WD, compared to both an HCD and traditional standard rat diet (SD). Diets were administered from weaning, throughout adolescence, and until early adulthood (P50, age of rat sexual maturity; Sengupta, 2013) to both male and female Long–Evans rats. Upon reaching early adulthood, calorie intake during diet administration, weight gain, anxiety-related and defensive behaviours in a modified OFT, along with CA3 hippocampal BDNF and plasma leptin levels, were measured. Importantly, the OFT, EPM, and LDB are all approach-avoidant anxiety tests, but subjecting rodents to all three tests does not increase

the chance of making a conclusion regarding anxiety levels (Crawley, 2007; Lezak et al., 2017; O'Leary et al., 2013). As such, researchers have attempted to mitigate these disparities and carry-over effects by using more complex behavioural testing apparatuses (e.g., the Multivariate Concentric Square Field™ Test developed by Meyerson et al., 2006) and have stressed the importance of adding additional measures of risk and exploration to testing paradigms (Gore-Langton et al., 2021). Thus, the traditional OFT (C. Hall & Ballachey, 1932) was modified for this study to incorporate the most salient measures of the OFT (i.e., center time, thigmotaxis) and include defensive behaviours traditionally measured in the EPM and LDB (e.g., hiding, stretch attends from a protected area). As previously described, WDs and HCDs have both been shown to increase anxiety-related behaviours, increase leptin, and decrease BDNF levels in rodents. Furthermore, WDs have been linked to elevated leptin levels and obesity, which are both related to increased anxiety. Thus, it was hypothesized that rats fed either the WD or HCD would show increased anxiety-related behaviours, lower BDNF levels, higher leptin levels, higher body weight, and higher caloric intake compared to the SD-fed rats.

2.3 Materials and Methods

2.3.1 Animals, Breeding, and Housing

All experimental procedures (see Figure 2.2) were approved by the Dalhousie University Committee on Laboratory Animals (protocol #19-028) and as per the guidelines of the Canadian Council on Animal Care. Male (226 – 250 g) and female (201 – 225 g) Long–Evans hooded rats were ordered for breeding (Charles River Laboratories, Raleigh, NC, USA). After two weeks of quarantine, rats were paired in two cohorts (one week apart) to produce offspring for the present experiment. During breeding, males were paired with a single female for seven days, after which the females were assumed to be pregnant, and the males were removed. Presumptively pregnant females were monitored for signs of pregnancy (i.e., pear-like shape, noticeable weight increase) and pair-housed until approximately four days before birth, when they were separated and housed singly.

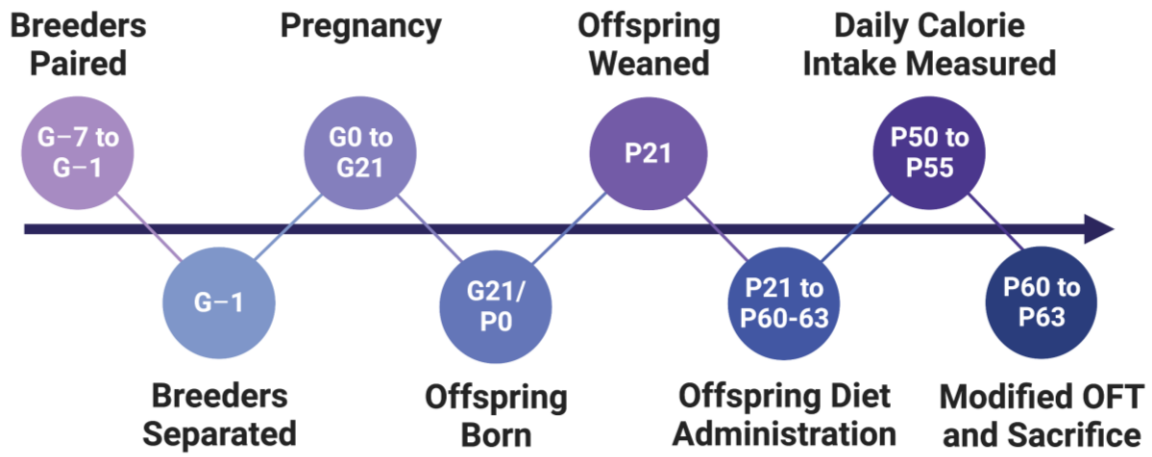


Figure 2.2 Experimental timeline from breeding to offspring sacrifices. G = gestational day (−7 – 21), P = postnatal day (0 – 63); OFT: open field test. Adapted from "Timeline (8 Segments, Horizontal)", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates. **Note: Fig. 1 in manuscript.**

From the first cohort of pregnant females, 26 offspring were used in this experiment, while 34 offspring from the second batch of pregnant females were used. At weaning (postnatal day 21, P21), offspring were separated into same-sex groups of two to three for housing and then randomly assigned to receive one of the three diets. The resulting sample sizes were: 20 in the standard diet (SD) group, 20 in the high-carbohydrate diet (HCD) group, and 20 in the Western diet (WD) group. More detailed information about the makeup of the experimental groups is provided in Table 2.1.

All rats were housed in polypropylene rat cages (47 cm × 24 cm × 20.5 cm) with wire lids. Cages were provisioned with softwood bedding (Shaw Resources, Shubenacadie, NS, Canada) and a black PVC tube (12 cm long, 8 cm diameter) for enrichment. Colony rooms were maintained at 20 °C ± 2 °C on a reversed 12:12 h light-dark cycle with lights off at 10:00. Breeder rats and preweaning offspring were provided with free access to SD (Laboratory Rodent Diet #5001, LabDiet, St. Louis, MO, United States) and all rats were provided free access to double-filtered municipal tap water.

Table 2.1 Litter characteristics for the six experimental groups, including sample size per group (total $N = 60$), number of litters that made up each experimental group, and maximum sample size per litter. SD: standard diet; HCD: high-carbohydrate diet; WD: Western diet; M: male; F: female. **Note: Table 1 in manuscript.**

	SD-M	**SD-F**	**HCD-M**	**HCD-F**	**WD-M**	**WD-F**
Sample size (*n*)	10	10	10	10	10	10
Contributing litters (*n*)	5	5	6	5	5	6
Max. *n* per litter	2	2	3	3	2	2

2.3.2 Offspring Diet Administration

Beginning on P21 until sacrifice (P60 – P63), offspring rats were provided with free access to one of the following: SD (cat. #5001, LabDiet®, St. Louis, MO, USA), HCD (cat. #D14042701, Research Diets, New Brunswick, NJ, USA), or WD (cat. #D12079B, Research Diets). Energy nutrient details for each diet are provided in Table 2.2, ingredient composition for the HCD and WD is provided in Supplementary Table S2.1, and the product sheet for the non-purified SD at the time it was used is presented as Supplementary Figure S2.1. The HCD is a commercially designed control diet for the WD. Overall, the WD has added cholesterol and is higher in saturated fat (anhydrous milk fat) and sucrose as compared to the HCD, which is higher in other carbohydrates (i.e., corn starch, maltodextrin) with no sucrose.

Table 2.2 The macronutrient breakdown in each diet, showing the percentage of energy (kcal) derived from fat, carbohydrates, and protein, along with the kilocalories per gram of food in each of the three diets used in this experiment. **Note: Table 2 in manuscript.**

	Standard Diet	**High-Carbohydrate Diet**	**Western Diet**
% Fat	13	10	40
% Carbohydrates	58	73	43
% Protein	29	17	17
Kcal/g	**3.4**	**3.9**	**4.7**

2.3.3 Body Weight Measurements and Average Daily Calorie Intake

Rats were weighed weekly at the exact ages of P21, P28, P35, P42, P49, P56, and on the day of sacrifice (P60 – P63). Because the day of sacrifice varied, early adult weight is reported as weight at age P56 in grams (g). A five-week weight gain measure (i.e., P21 – P56) is also included, as the weight taken at P56 would be the last weight measure before behavioural testing began. Between P50 and P55, available food (g) was measured (each 24h ± 15 mins) to calculate average food intake over a 5-day period. Then, the average daily food intake (g) for the cage was converted to absolute daily calorie intake (kcal) by multiplying the food intake by the kcal/g of that specific diet (see Table 2.2). This absolute cage calorie intake was divided by the number of rats per cage (i.e., 2 or 3) on the assumption that cages of two rats would split food 50:50 and cages of 3 rats would split food 33:33:33.

Because food intake is unlikely to be split exactly equally in the cages, a relative calorie intake value was also calculated for each rat based on P56 weight. Specifically, the absolute calorie intake was first normalized to P56 weight (i.e., absolute calorie intake divided by P56 weight). Then, for each cage, this normalized "kcal per body weight" value was used to determine the percent contribution of each rat to calorie intake based solely on their body weight (i.e., on the assumption that heavier rats would contribute more to the cage's calorie intake). The percent contribution value for each rat was then multiplied by the absolute cage calorie intake to yield a relative calorie intake value (i.e., both normalized to weight and relative to cage mates).

2.3.4 Modified Open Field Behavioural Testing

In a modified version of the OFT (mOFT; Figure 2.3; 80 cm × 80 cm × 35 cm), anxiety-related and defensive behaviours (see Table 2.3) were measured for 10-min under white light (age P60 – P63). Specifically, the light source was two 26W compact fluorescent light fixtures mounted to the ceiling 235 cm above the mOFT apparatus (General Electric, model number f26dbx/841/eco4p; 1440 mean lumens each). The black Plexiglas OFT (see Figure 2.3) was modified by including a refuge (i.e., hide box, 20 cm × 20 cm × 20 cm, 6 cm × 6

cm door) and adjusting the center area to account for the hide box walls being a perimeter. The hide box (floorless) was constructed from opaque black Plexiglas, except for one clear side to allow for viewing. A second closed black Plexiglas box was placed on top of the hide box to prevent rats from exiting the apparatus.

The floor of the mOFT was divided into a grid of 16 equal squares with Fisherbrand^{TM} tape (Thermo Fisher Scientific, Whitby, ON, Canada), with the center area being the inner three squares (18.75% of total floor space). At the start of each 10-minute test, rats were placed individually in the center of the mOFT, consistently facing one wall. Testing always occurred during the dark phase of the light-dark cycle at 30-minute intervals (i.e., two rats per hour). The first rat of the day was tested starting at 10:10, and the final rat was tested starting at 15:10 at the latest (depending on the exact number of rats tested on any given day). Line crosses, supported rearing, and unsupported rearing were live scored. Each test was also video recorded to allow for scoring of further behaviours. The mOFT was carefully cleaned with a 70% ethanol solution between trials and before the initial trial each day. After testing, rats were returned to their home cage with free access to food and water before sacrifice.

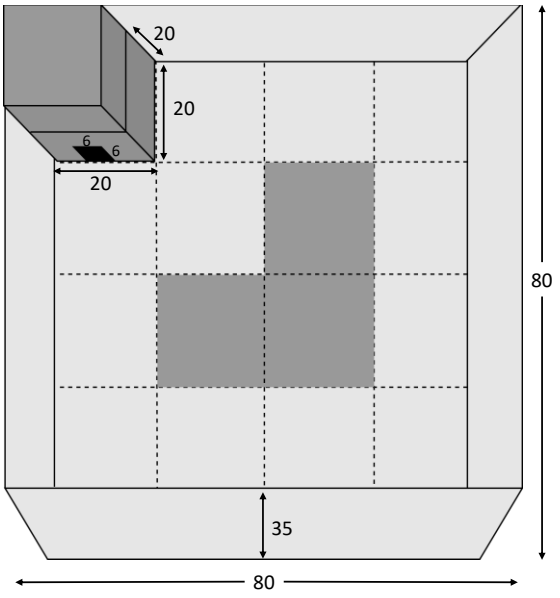


Figure 2.3 Schematic (in centimeters) of the modified open field test (i.e., with hide box, adjusted center area in darker gray) used in this experiment for the 10-min behavioural test. **Note: Fig. 2 in manuscript.**

Table 2.3 Ethogram of behaviours measured in the modified open field test. Behaviours were interpreted by the following categories: ↓A/L = lower anxiety or locomotion; ↓A/E = lower anxiety or exploration; ↑A = higher anxiety behaviour. **Note: Table 3 in manuscript.**

Behaviour	Operational Definition	Reference
Line Crosses ↓A/L	Frequency of all four paws crossing into a new quadrant, straight or diagonally (not including entries into hide box)	(Korgan et al., 2016)
Supported Rearing[a] ↓A/L	Frequency of standing on hind legs with a vertically extended body; one or both paws touching any wall	(Sturman et al., 2018)
Unsupported Rearing[a] ↓A/E	Frequency of standing on hind legs with vertically extended body without support	(Sturman et al., 2018)
Time in Center ↓A/E	Duration (s) with all four paws residing within marked center area	(Kalueff & Tuohimaa, 2004)
Latency from Center ↑A	Time (s) taken for all four paws to be outside center area after initial placement	(Kalueff & Tuohimaa, 2004)
Center Entries ↓A/E	Frequency of times the rat enters the marked center of the open field with all four paws	(Kalueff & Tuohimaa, 2004)
Time in Hide Box ↑A	Duration (s) spent with all four paws inside hide box	(Choleris et al., 2001)
Head Outs from Hide Box ↓A/E	Frequency of rat peeking head out from hide box opening while remaining in hide box	(Choleris et al., 2001)
Hide Box Entries ↑A	Frequency of all four paws entering hide box	(Choleris et al., 2001)
Thigmotaxis ↑A	Duration (s) spent in physical contact (i.e., side of the abdomen) with a perimeter or hide box wall while in motion or still	(Joshi et al., 2017)
Hide Box Entry Latency ↓A/E	Time (s) taken to first entry of hide box	N/A

Behaviour	Operational Definition	Reference
Latency to Re-Enter Center ↑A	Time (s) taken to re-enter center area after leaving following initial placement	(Kalueff & Tuohimaa, 2004)
Stretch Attend Postures ↑A	Frequency of rat stretching forward and flattening the back in an exploratory manner	(Ortolani et al., 2011)
General Risk Assessment ↑A	Frequency of exits from the periphery of the mOFT to the center with only the front two paws and head the center	(Ortolani et al., 2011)

[a]To count as two rearing events, rats must touch with one or both front paws between events.

2.3.5 Sacrifice and Tissue Collection

Rats were anesthetized with Euthanyl (sodium pentobarbital, DIN 00141704) 60 min after concluding behavioural testing as per the protocol in Franklin and Perrot-Sinal (2006). Thus, all sacrifices occurred during the dark phase of the light-dark cycle at specific 30-min intervals (i.e., two rats per hour). Specifically, the first rat of the day was sacrificed starting at 11:20 (60 mins after concluding behavioural testing), and, at maximum, the final rat was sacrificed starting at 14:20. Once deep anesthetization was confirmed by the lack of a toe pinch reflex, rats were decapitated by guillotine. Trunk blood was collected immediately into microtubes with 20 µL of heparin (Heparin Leo, Leo Pharma, DIN 00727520, 100 IU/mL) and placed on ice. Plasma was prepared by centrifuging the whole blood at 4 °C for 15 min at 1,000 *g*, followed by 4 °C for 10 mins at 10,000 *g*. The supernatant (plasma) was stored at −80 °C until assay. Whole brains were removed, flash-frozen in chilled isopentane (around −70 °C) and stored at −80 °C until further processing.

2.3.6 Hippocampal Tissue Protein Extraction

Using a cryostat (CM1850UV, Leica Biosystems Inc., Concord, ON, Canada), 600 µm sections of whole brains were coronally dissected (−3.2 to −3.8 mm from Bregma) based on the protocol from Franklin and Perrot-Sinal (2006) and using Paxinos and Watson (1986) as a guide. Specifically, with an 18-gauge blunted needle, bilateral micropunches

of the CA3 region of the hippocampus were taken and stored at −80 °C until protein extraction.

To prepare the needed 20 mL cell lysis buffer cocktail for protein extraction, the Bio-Plex cell lysis kit (cat. #171304011; Bio-Rad Laboratories, Mississauga, ON, Canada) was used. Specifically, 80 µL of cell lysis buffer factor 1 (Bio-Plex cell lysis kit), 40 µL of cell lysis buffer factor 2 (Bio-Plex cell lysis kit), and 80 µL of 500 mM phenylmethylsulfonyl fluoride (PMSF) in dimethyl sulfide (DMS) were added to 19.8 mL of cell lysis buffer (Bio-Plex cell lysis kit). To prepare 1 mL of 500 mM PMSF (cat. #P7626250mg, Sigma-Aldrich Canada Co., Oakville, ON, Canada), 0.0871 g of PMSF was added to 1 mL of DMS (cat. #D2650-100mL, Sigma-Aldrich). During protein extraction, 300 µL of prepared cell lysis buffer was added to each microtube containing the bilateral CA3 micropunches. After the cell lysis buffer cocktail was added, samples were sonicated (cat. #Q800R2, Qsonica, Newtown, CT, USA) with settings of 2 × 30 s at 4 °C and 60% amplitude. An aliquot was used to determine protein concentration using Bradford assay in duplicate (Bradford Reagent, cat. #B6916-500mL, Sigma-Aldrich), with a standard curve of bovine serum albumin (cat. #A2153-10G, Sigma-Aldrich).

2.3.7 Brain-Derived Neurotrophic Factor (BDNF) Concentration

BDNF concentration (pg/mL) in the micropunches was determined by Enzyme-Linked Immunosorbent Assay (ELISAs; cat. #ERBDNF, lot #750111-819, Thermo Fisher Scientific, Life Technologies Corporation, Frederick, MD, USA) following manufacturer's instructions. Samples were diluted to 1 in 2 based on a dilution series optimization and assayed in duplicate. Interassay and intraassay variability are reported by the manufacturer as < 12% and < 10%, respectively. Two BDNF ELISA plates were run, with $R^2 = 0.997$ and 0.998, while counterbalancing for diet type and sex. Ten samples on the first plate had CVs above 20%, and, therefore, were re-run on the second plate, where they were all found to have CVs of less than 20%. BDNF concentration was normalized to total protein (µg/µL) levels. Results are reported as pg BDNF per µg total protein.

2.3.8 Leptin Concentration

Plasma leptin concentrations (pg/mL) were determined using a leptin ELISA kit (cat. #KRC2281, lot #253885-001, Thermo Fisher Scientific, Life Technologies Corporation, Frederick, MD, USA), following manufacturer's instructions. Samples were diluted to 1 in 10 based on a practice dilution series and assayed in duplicate while counterbalancing for diet type and sex. Interassay and intraassay variability are reported by the manufacturer as 4.6% and 3.6%, respectively. Two plates were run, each having a standard curve with an $R^2 = 1$. Two samples from the first plate were found to have CVs greater than 10% (a lower cut-off because other duplicates had such low CVs), and, therefore, were re-assayed on the second plate and found to have CVs below 10%.

2.3.9 Statistical Analyses

All statistical analyses were conducted using jamovi (Version 2.3.0; Fox & Weisberg, 2020; Lenth, 2020; R Core Team, 2021; The jamovi project, 2022). GraphPad Prism (Version 9.2.0; GraphPad Software, San Diego, CA, USA) was used to create graphs. Most behavioural, along with all weight (P21, P56, and weight gain), food and calorie intake (absolute and relative), leptin, and CA3 BDNF dependent variables were analyzed using 2 (Sex; male, female) × 3 (Diet; SD, HCD, and WD) factorial analyses of variance (ANOVAs). mOFT behaviours related to locomotion (i.e., line crosses, supported rearing; see Table 3) were analyzed using 2 (Sex; male, female) × 3 (Diet; SD, HCD, and WD) factorial analyses of covariance (ANCOVAs), with P56 weight as a covariate. Pearson's correlations were conducted between specific metabolic measures (i.e., weight gain, absolute caloric intake, CA3 BDNF, leptin) by experimental group. Pearson's correlations by experimental group were also conducted between significantly different behavioural variables and between these behavioural variables and the aforementioned metabolic variables. The alpha level was set at 0.05 for all analyses, with effect sizes reported as eta squared (η^2). As summarized by Lakens (2013), J. Cohen (1988) defines a small effect at $\eta^2 = 0.01$, a medium effect at $\eta^2 = 0.06$, and a large effect at $\eta^2 = 0.14$. *Post hoc* testing of main effects of diet and significant interactions were conducted with Tukey's *post hoc* test.

The final sample size was 60, as previously described (Table 2.1). All dependent variables were checked for outliers. Outliers were identified if they were greater or less than three standard deviations (*SD*) from the overall mean (*M)*, and a decision was made to keep or remove them based on the type of variable (Dunn, 2021). Specifically, the upper limit was calculated as [*M* + (3**SD*)] and the lower limit was calculated as [*M* − (3**SD*)] (Dunn, 2021). Homogeneity of variance was analyzed and if variance was not homogenous (i.e., Levene's test was significant), then a generalized linear model was conducted (IBM SPSS Statistics, version 28.0.1.1) to confirm ANOVA findings (summarized in Appendix C).

Specifically, for behavioural analyses, one rat (Male-SD) was removed from analyses as the rat did not move during the 10-minute test, leaving a final sample size of 59 for the anxiety-related and defensive behaviours. A further nine behavioural outliers were identified as being greater than 3 *SD*s from the mean and were removed from final data analysis for those specific dependent variables. These outliers included one value for total unsupported rearing (Male-HCD), one for time in center (Male-HCD), one for latency from center (Male-HCD), one for center entries (Male-SD), one for time in hide box (Female-WD), one for thigmotaxis (Female-WD), two for center re-entry latency (Male-WD, Female-WD), and one for stretch attend postures (Female-WD). For normalized CA3 BDNF levels, one value (Male-HCD) was greater than 3 *SD*s from the mean, but since the value met other equally stringent criteria (i.e., concentration was within the standard curve for the ELISA and the sample had a CV between duplicates of < 20%), this value remained in analyses. For plasma leptin levels, there was also one value (Male-WD) greater than 3 *SD*s from the mean, but this value was similarly retained as it fell within the standard curve on that ELISA and had a CV of less than 20% between duplicates. There were no outliers found for P21 weight, P56 weight, weight gain, food intake, or calorie intake measures.

2.4 Results

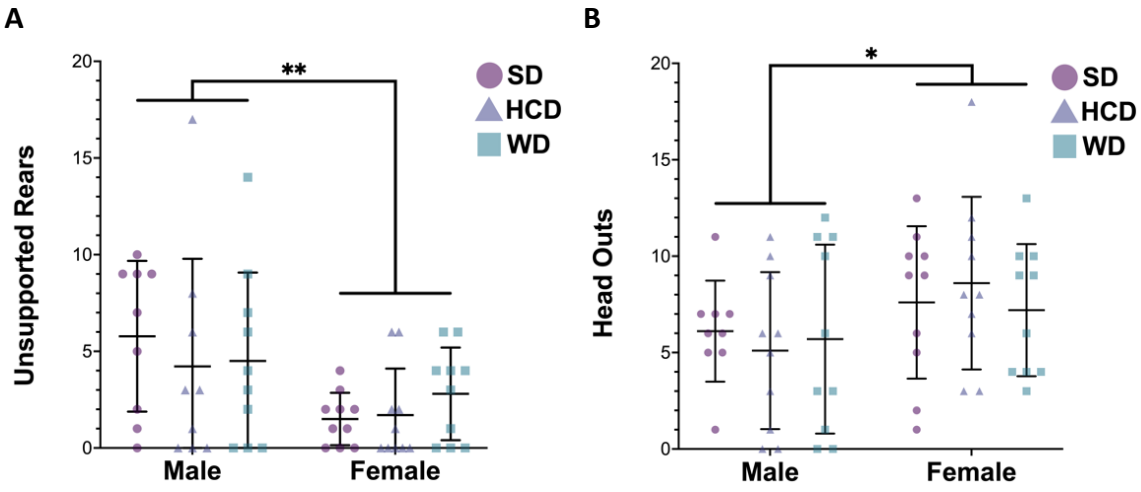
2.4.1 Anxiety-Related Behaviours

A series of 12 sex by diet (2 × 3) factorial ANOVAs did not reveal any main effects or interactions for the following anxiety-related and defensive behaviours: time in center,

latency from center, center entries, time in hide box, hide box entries, thigmotaxis, hide box entry latency, latency to re-enter center, and general risk assessment (Supplementary Table S2.2). Furthermore, two sex by diet (2 × 3) factorial ANCOVAs (P56 weight as a covariate) did not reveal any main effects or interactions for line crosses or supported rearing (Supplementary Table S2.2). Descriptive statistics for all 14 behavioural variables are included as Supplementary Table S2.3 (by sex; male and female) and Supplementary Table S2.4 (by diet; SD, HCD, and WD).

The sex by diet (2 × 3) factorial ANOVAs did reveal a main effect of sex for unsupported rearing ($F_{1,52} = 8.91$, $p = .004$, $\eta^2 = 0.142$) and head outs ($F_{1,53} = 4.32$, $p = .043$, $\eta^2 = 0.074$). Specifically, males engaged in more unsupported rearing than females (Figure 2.4A), and females engaged in more head outs from the hide box than males (Figure 2.4B).

There was also a main effect of sex for stretch attend postures ($F_{1,52} = 9.05$, $p = .004$, $\eta^2 = 0.128$) with males ($M = 7.86$; $SD = 3.83$) performing more stretch attend postures than females ($M = 4.83$; $SD = 4.12$). However, a sex by diet interaction ($F_{2,52} = 4.76$, $p = .013$, $\eta^2 = 0.135$) with Tukey's *post hoc* comparisons showed that it was males in the WD group only that performed more stretch attend postures compared to females in the WD group ($p = .028$; Figure 2.4C). There were no sex differences in stretch attend postures in SD ($p = .970$) or HCD ($p = .068$) animals.



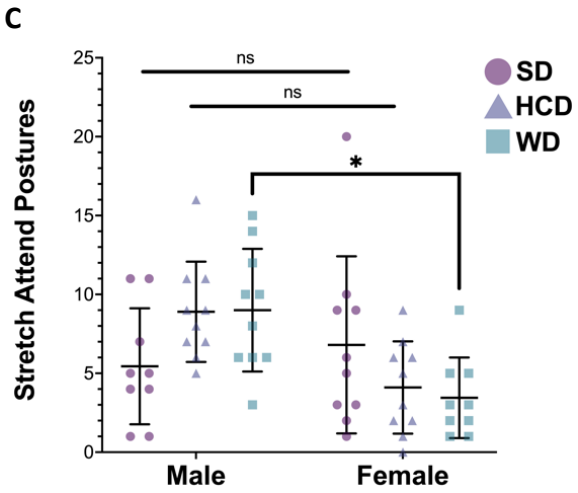


Figure 2.4 Graphical overview of **A.** The main effect of sex on unsupported rears, **B.** The main effect of sex on head outs from the hide box, and **C.** The interaction between sex and diet for stretch attend postures during modified open field testing. *$p < .05$, **$p < .01$; ns = not significant, SD = standard diet, HCD = high-carbohydrate diet, WD = Western diet; data expressed as $M \pm SD$. **Note: Fig. 3 in manuscript.**

2.4.2 Weight and Calorie Intake Measures

A sex by diet (2 × 3) factorial ANOVA on wean (P21) weight at the start of diet administration showed that wean weight of animals was not significantly affected by sex ($p = .267$), diet ($p = .349$), or the sex by diet interaction ($p = .161$; Figure 2.5A). At P56, animals were weighed to provide early adulthood body weight. A sex by diet (2 × 3) factorial ANOVA revealed a main effect of sex ($F_{1,54} = 283.35$, $p < .001$, $\eta^2 = 0.768$) and diet ($F_{2,54} = 14.27$, $p < .001$, $\eta^2 = 0.077$). By sex, males ($M = 339.44$; $SD = 32.99$) weighed more than females ($M = 240.89$; $SD = 20.73$). Tukey's *post hoc* analyses on the main effect of diet showed that while the HCD and WD groups were not different in adult weight ($p = .105$), the SD group ($M = 269.79$; $SD = 46.17$) weighed less than both the HCD ($M = 292.92$; $SD = 58.64$; $p = .006$) and the WD groups ($M = 307.79$; $SD = 60.35$; $p < .001$; Figure 2.5B).

In addition to the absolute weight in early adulthood (P56), weight gain from weaning (i.e., five weeks on diets) was also calculated. A sex by diet (2 × 3) factorial ANOVA revealed

a main effect of sex ($F_{1,54}$ = 326.25, p < .001, η^2 = 0.785) and diet ($F_{2,54}$ = 16.75, p < .001, η^2 = 0.081). By sex, males (M = 293.94; SD = 30.62) gained more weight than females (M = 197.22; SD = 19.69). Tukey's *post hoc* analyses showed that while the HCD and WD groups did not differ in five-week weight gain (p = .168), the SD group (M = 224.79; SD = 46.16) weighed less than both the HCD (M = 249.96; SD = 56.25; p < .001) and the WD groups (M = 261.99; SD = 57.91; p < .001; Figure 2.5C).

Prior to converting to calorie intake, food intake (g) per rat (P50 – P55 average) was analyzed by a sex by diet (2 × 3) ANOVA. The ANOVA revealed main effects of sex ($F_{1,54}$ = 84.65, p < .001, η^2 = 0.447) and diet ($F_{2,54}$ = 25.21, p < .001, η^2 = 0.266). Overall, males (M = 29.34; SD = 3.77) ate more by food weight (g) than females (M = 22.89; SD = 3.53). Tukey's *post hoc* test on the main effect of diet revealed that the SD group (M = 29.59; SD = 4.06) consumed more food by weight than the HCD (M = 24.82; SD = 4.67; p < .001) and WD groups (M = 23.92; SD = 3.96; p < .001) and the HCD and WD groups did not differ in weight of food eaten (p = .550; Figure 2.5D).

A further sex by diet (2 × 3) factorial ANOVA on absolute calorie intake was conducted and revealed a main effect of sex ($F_{1,54}$ = 80.33, p < .001, η^2 = 0.513) and diet ($F_{2,54}$ = 10.83, p < .001, η^2 = 0.138). By sex, males (M = 116.08; SD = 13.23) consumed more average daily calories than females (M = 90.47; SD = 12.10). Tukey's *post hoc* comparisons showed that the SD and HCD groups were not different in daily calories consumed (p = .523). However, the WD group (M = 112.42; SD = 18.63) consumed more daily calories than the SD (M = 100.62; SD = 13.79; p = .004) and HCD groups (M = 96.80; SD = 18.20; p < .001; Figure 2.5E). Relative calorie intake results paralleled those of absolute calorie intake, whereby the sex by diet (2 × 3) ANOVA also revealed a main effect of sex ($F_{1,54}$ = 70.48, p < .001, η^2 = 0.489) and diet ($F_{2,54}$ = 9.59, p < .001, η^2 = 0.133). Males (M = 115.84; SD = 14.02) again consumed more calories than females (M = 90.40; SD = 12.37) when considering relative consumption based on P56 weight in each cage. Furthermore, Tukey's *post hoc* test still revealed that the WD group (M = 112.23; SD = 19.08) consumed more calories than the SD (M = 100.52; SD = 14.25; p = .007) and HCD (M = 96.61; SD = 18.39; p < .001) rats, with no difference between SD and HCD groups (p = .546; Figure 2.5F).

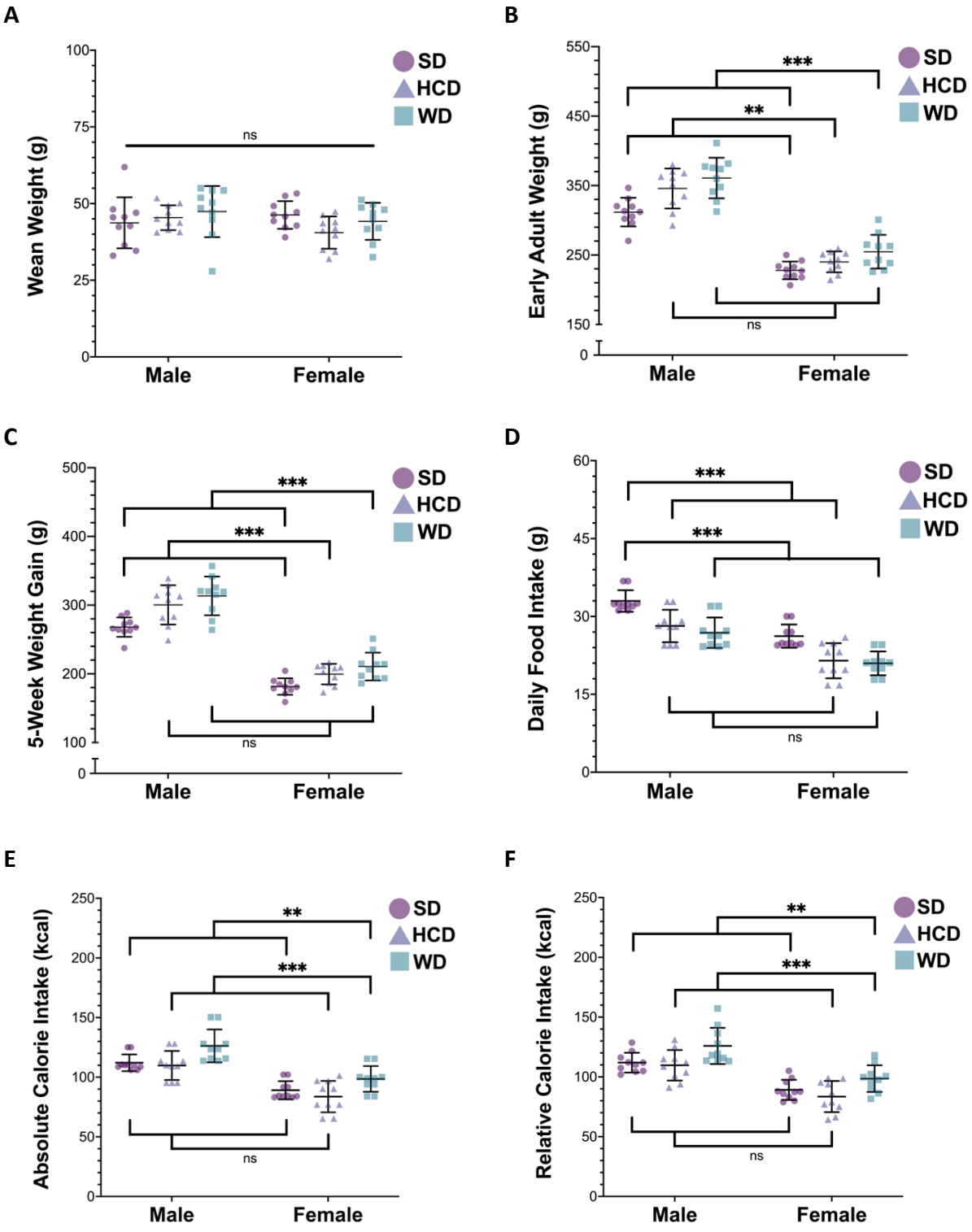


Figure 2.5 Graphical overview **A.** Wean (P21) weight (g), **B.** The main effect of diet on early adult (P56) weight (g), **C.** The main effect of diet on weight gain (g) during diet administration (P21 – 56), **D.** The main effect of diet on average daily food intake (g; P50 – 55), **E.** The main effect of diet on

absolute calorie intake, and **F.** The main effect of diet on relative calorie intake. $^{**}p < .01$, $^{***}p < .001$; ns = not significant, SD = standard diet, HCD = high-carbohydrate diet, WD = Western diet; data expressed as $M \pm SD$. *Note*: to simplify figures, sex main effects are not depicted in these figures as they are described above and always higher in males compared to females. **Note: Fig. 4 in manuscript.**

2.4.3 Plasma Leptin and Hippocampal Brain-Derived Neurotrophic Factor Concentrations

Another factorial sex by diet (2 × 3) ANOVA on plasma leptin concentration revealed a main effect of sex ($F_{1,54} = 22.25$, $p < .001$, $\eta^2 = 0.182$) and diet ($F_{2,54} = 19.77$, $p < .001$, $\eta^2 = 0.323$). By sex, males ($M = 7294.30$; $SD = 5179.16$) had higher leptin levels compared to females ($M = 3633.92$; $SD = 2092.90$). By diet, Tukey's *post hoc* comparisons showed that the HCD and WD groups were not different in leptin levels ($p = .196$). Nevertheless, the SD group ($M = 2150.13$; $SD = 795.04$) did have lower leptin than both the HCD ($M = 6289.10$; $SD = 3405.59$; $p < .001$) and WD groups *(*$M = 7953.10$; $SD = 5213.11$; $p < .001$).

These main effects were superseded by a significant sex by diet interaction effect on leptin levels ($F_{2,54} = 3.32$, $p = .044$, $\eta^2 = 0.054$). Tukey's *post hoc* comparisons showed that leptin levels in females were not affected by diet, but in males, the SD group had lower levels compared to both the HCD males ($p = .002$) and WD males ($p < .001$). Furthermore, while males and females were not different in leptin in the SD group ($p = .967$), males had higher levels of leptin than females in the HCD ($p = .0497$) and WD groups ($p < .001$; Figure 2.6A).

Finally, a sex by diet (2 × 3) factorial ANOVA revealed a main effect of sex on normalized BDNF levels in the CA3 region ($F_{1,54} = 4.24$, $p = .044$, $\eta^2 = 0.068$) in that males ($M = 0.203$; $SD = 0.135$) had higher BDNF in this region relative to total protein levels compared to females ($M = 0.143$; $SD = 0.086$; Figure 2.6B).

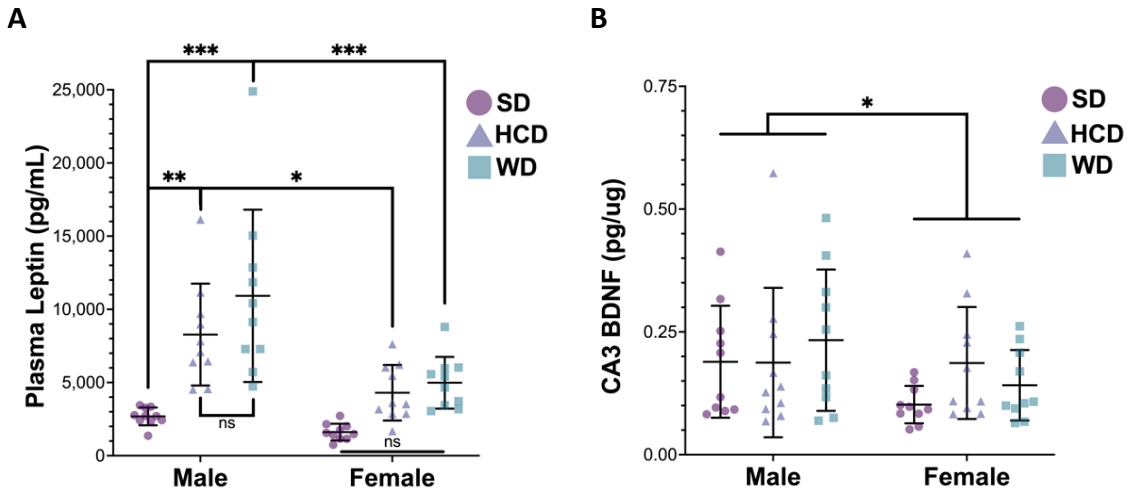


Figure 2.6 Graphical overview of **A.** The sex by diet interaction on plasma leptin levels, and **B.** The main effect of sex for normalized brain-derived neurotrophic factor (BDNF) in the CA3 region of the hippocampus. *$p < .05$, **$p < .01$, ***$p < .001$; ns = not significant, SD = standard diet, HCD = high-carbohydrate diet, WD = Western diet; data expressed as $M \pm SD$. **Note: Fig. 5 in manuscript.**

2.4.4 Correlations Between Behavioural and Metabolic Variables

Pearson's correlations showed that many of the metabolic variables were significantly positively correlated, indicating that higher levels of certain metabolic variables are related to higher levels of other variables (Table 2.4). No significant negative correlations were observed between these variables. Weight gain was included in place of P56 weight to simplify results as these two variables were correlated above 0.935 in all subsets analyzed. Furthermore, calorie intake refers to absolute calorie intake. Specifically, when including all rats in the experiment, calorie intake was positively correlated with weight gain, leptin, and CA3 BDNF. Furthermore, weight gain was positively correlated with leptin and CA3 BDNF, whereas CA3 BDNF and leptin were not significantly correlated.

When males were separated out, calorie intake was again positively correlated with weight gain and leptin but was no longer significantly correlated with CA3 BDNF. As well, weight gain and leptin remained positively correlated, but CA3 BDNF and leptin were still not significantly correlated. Finally, in males, weight gain and CA3 BDNF were not

significantly correlated. In females, the only significant correlations found were positive correlations between weight gain and leptin and weight gain and CA3 BDNF. In SD animals, calorie intake was positively correlated with weight gain and leptin, and weight gain was positively correlated with both leptin and CA3 BDNF. No significant correlations were found with CA3 BDNF and calorie intake or leptin. Results for HCD animals paralleled those of males in that the only significant correlations found were positive correlations between calorie intake and weight gain, calorie intake and leptin, and weight gain and leptin. For both males and HCD animals, no significant correlations were found with CA3 BDNF. In WD animals, positive correlations were found for calorie intake and weight gain, calorie intake and leptin, and weight gain and leptin. In these animals, CA3 BDNF was not correlated with calorie intake or weight gain, but this group was the only group where CA3 BDNF and leptin were positively correlated.

When breaking groups down further by both sex and diet, certain positive correlations were found to be significant in males given SD, females given HCD, males given WD, and females given WD. Specifically, in SD males, calorie intake and leptin were positively correlated. In contrast, in HCD females, calorie intake and weight gain, along with weight gain and leptin were positively correlated. In WD males, calorie intake and leptin were positively correlated, whereas, in WD females, weight gain and CA3 BDNF were positively correlated.

Table 2.4 Pearson's correlations between absolute calorie intake and weight gain, absolute calorie intake and leptin, weight gain and leptin, CA3 BDNF and absolute calorie intake, CA3 BDNF and weight gain, and CA3 BDNF and leptin in all rats ($N = 60$), along with subsets by sex and diet ($n = 10 – 30$). **Note: Table 4 in manuscript.**

Subset (*n*)	**Calorie Intake & Weight Gain**	**Calorie Intake & Leptin**	**Weight Gain & Leptin**	**CA3 BDNF & Calorie Intake**	**CA3 BDNF & Weight Gain**	**CA3 BDNF & Leptin**
All rats (60)	0.778***	0.592***	0.635***	0.267*	0.300*	0.252
By sex						
M (30)	0.499**	0.570**	0.624***	0.174	0.042	0.187
F (30)	0.360	0.253	0.622***	0.028	0.428*	0.072

Subset (*n*)	Calorie Intake & Weight Gain	Calorie Intake & Leptin	Weight Gain & Leptin	CA3 BDNF & Calorie Intake	CA3 BDNF & Weight Gain	CA3 BDNF & Leptin
By diet						
SD (20)	0.831***	0.816***	0.669**	0.305	0.457*	0.350
HCD (20)	0.844***	0.638**	0.648**	0.146	0.003	-0.190
WD (20)	0.756***	0.693***	0.668**	0.388	0.435	0.487*
By sex and diet						
SD M (10)	0.100	0.733*	0.306	-0.198	0.153	-0.028
SD F (10)	0.009	0.472	-0.327	-0.421	-0.562	0.242
HCD M (10)	0.552	0.282	0.217	0.196	-0.142	-0.210
HCD F (10)	0.879***	0.559	0.636*	0.238	0.366	-0.319
WD M (10)	0.412	0.693*	0.462	0.144	-0.001	0.423
WD F (10)	-0.104	-0.294	0.279	0.175	0.796**	-0.131

*$p < .05$, **$p < .01$, ***$p < .001$; M = male, F = female, SD = standard diet, HCD = high-carbohydrate diet, WD = Western diet, BDNF = brain-derived neurotrophic factor.

Pearson's correlations between behavioural variables found to be significantly different by ANOVA (i.e., unsupported rearing, head outs, stretch attend postures) revealed notable findings. When examining all rats, head outs and stretch attend postures were found to be negatively correlated (r = -0.434, $p < .001$, n = 58; Supplementary Table S2.5). Furthermore, unsupported rearing and weight gain were found to be positively correlated (r = 0.261, $p < .05$, n = 58; Supplementary Table S2.6), no correlations were found with head outs and metabolic measures (Supplementary Table S2.7), and stretch attend postures and weight gain were positively correlated (r = 0.313, $p < .05$, n = 58; Supplementary Table S2.8).

Examining correlations by sex, diet, and their specific group combinations revealed distinct significant relationships between metabolic and behavioural variables that are summarized in Figure 2.7 and presented in Supplementary Tables S2.5 to S2.8. Specifically, head outs and stretch attend postures were negatively correlated in males and stretch attend postures were negatively correlated with leptin levels in females. Importantly, all three key behavioural variables were found to be interrelated in WD animals and stretch attend postures were positively correlated with weight gain in this group. Unsupported rearing

was positively correlated with calorie intake, weight gain, and leptin in SD animals. Furthermore, stretch attend postures were positively corelated with weight gain and leptin in HCD rats.

Additional distinct relationships between metabolic and behavioural variables were found when examining correlations in each of the six experimental groups (Figure 2.7). Between behavioural variables, head outs were negatively correlated with stretch attend postures in HCD females but negatively correlated with unsupported rearing in WD males. Including metabolic variables, unsupported rearing was found to be negatively associated with CA3 BDNF levels in SD males, positively associated with leptin in HCD females, and negatively associated with calorie intake in WD males. Interestingly, the only significant correlations found between head outs and metabolic variables were in WD males. In this specific group, head outs were positively correlated with both calorie intake and weight gain. In HCD females, stretch attend postures were negatively associated with leptin but were positively associated with leptin in HCD males. Finally, stretch attend postures and calorie intake were positively correlated only in SD females.

2.5 Discussion

The objective of the present study was to provide a comparative analysis of specific physiological and psychological effects of a Western diet (WD) compared to two control diets, whose use is currently debated in the literature (e.g., Warden & Fisler, 2008). We directly compared the effects of a WD to one of its commercially available control diets (HCD; i.e., a diet that is, by design, unavoidably high in carbohydrates) and a standard diet (SD; i.e., a commonly used rodent lab chow that is not as rigorously characterized as commercially available purified diets). We included an equivalent number of males and females in each diet group to delineate which effects might be sex-specific. Our physiological outcome measures were hippocampal CA3 BDNF, plasma leptin, calorie intake (absolute and relative), and weight (P21 weight, P56 weight, and P21 – P56 weight gain). Our psychological variables were specific anxiety-related and defensive behaviours measured in a modified OFT (i.e., with a hide box and behaviours not usually analyzed in a traditional OFT).

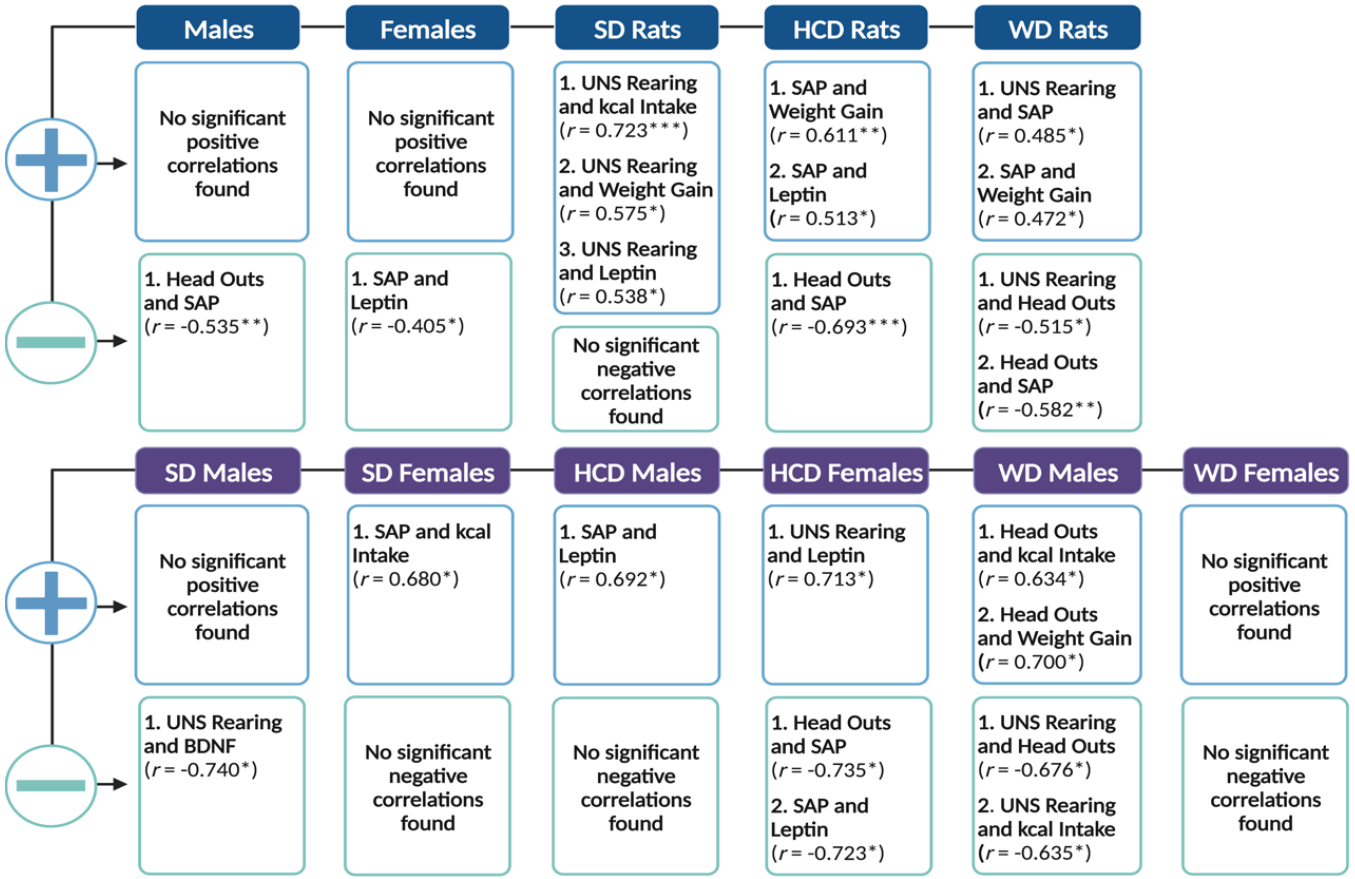


Figure 2.7 Summary of significant correlations between key behavioural (i.e., unsupported rearing, head outs, stretch attend postures) and metabolic variables (i.e., absolute calorie intake, weight gain, leptin, CA3 BDNF) in all rats (n = 59) and sex and diet subsets (n = 9 – 30). *p < .05, **p < .01, ***p < .001; SD = standard diet, HCD = high-carbohydrate diet, WD = Western diet, BDNF = brain-derived neurotrophic factor, kcal = calorie, UNS = unsupported, SAP = stretch attend posture. Adapted from "Flow Chart (5 Levels, Vertical) 7", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates. **Note: Fig. 6 in manuscript.**

Despite no significant effects of diet on anxiety-related measures, in animals that received the WD, males performed more stretch attend postures (a behaviour indicative of higher anxiety) than females. Similarly, Panetta et al. (2017) showed in the EPM that male Sprague–Dawley rats demonstrated more anxiety-related behaviours when administered 60% HFD compared to females, who were seemingly less affected by diet in their presentation of anxiety in the EPM. Still, available rodent literature remains mixed on how a WD (or HFD) may influence anxiety and how this relationship may be different for each sex. For instance, with female Long–Evans rats, one study reported more anxiety-related behaviours in the LDB and OFT after ten weeks of a 60% HFD compared to a 70% carbohydrate control diet (Sivanathan et al., 2015). In contrast, Ohland et al. (2016) showed that male mice exhibit lower anxiety-related behaviours in a latency to step down test after three weeks of a WD (33% fat; 49% carbohydrates) compared to an SD (LabDiet® 5001). At present, no conclusions can be drawn from these disparate results as there are too many methodological differences (e.g., different species, tests of anxiety, control diet used, sex, length of diet exposure). Nevertheless, research does suggest that HFDs and WDs exert a bidirectional effect on anxiety symptoms (i.e., shorter-term consumption reducing anxiety, and longer-term consumption promoting anxiety; Sweeney et al., 2017). Unfortunately, the present results do not provide any more clarity in this area, and further work is still needed.

Results of this study do highlight significant overall sex differences related to exploration and anxiety during behavioural testing. Specifically, males performed more unsupported rearing than females, and females performed more head outs from the hide box than males. Both behaviours are related to exploration, albeit females demonstrated more exploration (via head outs) from the safety of a refuge area, whereas males demonstrated increased exploration (via unsupported rear) in general. Previous work has shown that females rear less than males and that unsupported rearing, at least in male mice, is indicative of lower anxiety (Sturman et al., 2018). Head outs are a behaviour more commonly attributed to the LDB or EPM, whereby the rodent extends its head into a more anxiety-inducing location (i.e., light, or open arm, respectively) without leaving the lower-anxiety location (i.e., dark, or closed arm, respectively; Maestas-Olguin et al., 2021). Dielenberg and McGregor (2001) describe head outs as a monitoring behaviour, distinct from the exploration that is typically

associated with rearing, which highlights that it may be important to measure when possible. Interestingly, head outs (a low anxiety exploratory behaviour; Table 2.3) and stretch attend postures (a high anxiety exploratory behaviour; Table 2.3) were negatively correlated in males but not in females. Nevertheless, there are specific limitations to the behavioural testing paradigm in this study that are important to acknowledge. First, even though our chosen measure of anxiety-related and avoidance behaviours was detailed in its analysis, certain decisions (e.g., modifying the center area, using a hide box) may warrant further detailed analysis by comparing directly to a traditional open field. Our goal with the one chosen behavioural test was to help mitigate some of the limitations with repeated testing and inconsistent findings (described in detail by O'Leary et al., 2013) by incorporating more specific measures of anxiety and avoidance into one test. In saying this, because the mOFT was a modification of a traditional OFT, generalizability of specific results is limited, and sex effects might differ if a different behavioural test is used. Relatedly, as the behavioural testing component of this experiment was live- and video-scored manually by observers, more advanced scoring set-ups, whereby variables such as average speed or total distance travelled could be included, would increase the amount of detailed information we can ascertain from testing.

Unsurprisingly, males weighed more and gained more weight than females in early adulthood. In support of our hypotheses, males and females fed the standard diet weighed the lowest at P56 and gained the least weight compared to the WD group, while HCD and WD groups did not differ in these measures. Considering that this specific HCD is a commercially available control diet for this particular WD, it is noteworthy that the weight of rats did not differ, even after diet administration from P21 to P56 (i.e., five weeks). Previous work by S. Lin et al. (2000) compared HFD and HCD control diets in C57BL/6J male mice starting at weaning and showed that even after two weeks of diet administration, body weight was significantly increased in the HFD group, and this finding persisted through the 19-week study. In the present work, differences in weight may have appeared with longer diet administration or, possibly, young rats are not as sensitive to the WD as other young rodents, which would limit differences in body weight between WD and HCD groups at this age (P56).

Consistent with the sex difference in weight gain, males consumed more daily calories compared to females. However, dissimilar to diet-induced differences in weight gain, the WD group consumed more calories than both the HCD and SD groups, which did not differ in their calorie intake. In previous work, S. Lin et al. (2000) reported higher calorie consumption after a 19-week HFD exposure (59% kcal fat, 14% kcal carbohydrates) when compared to an HCD control (10% kcal fat, 63% kcal carbohydrates). Similarly, Ortolani et al. (2011) observed higher caloric intake after just five days of comfort foods (e.g., chocolate, cookies; 20% kcal fat, 48% kcal carbohydrates) compared to a conventional lab chow (an SD). Although these experimental diets are not analogous in their composition, one broad explanation for increased caloric intake with free access to unhealthy diets (e.g., WDs, HFDs) is that more palatable diets activate the brain's reward system to release dopamine after consumption (Licholai et al., 2018). Interestingly, the HCD and SD groups did not differ in calorie intake, despite weight gain being higher in the HCD group. In contrast to our findings, Bursać et al. (2014) showed that fructose-administered adult male Wistar rats had increased calorie intake and increased mass of visceral omental adipose tissue compared to rats that were administered an SD, despite total body weight being similar between groups (Bursać et al., 2014). As previously mentioned, S. Lin et al. (2000) documented that at two weeks after beginning administration (at weaning), HFD-fed mice consistently had higher body weight than HCD-fed mice, but, interestingly, calorie intake between the two groups at each time interval followed a less predictable pattern. Although calorie intake between the two groups was similar for the first four weeks, the HFD mice then started to decrease their calorie intake, but then increase it dramatically after 15 weeks (S. Lin et al., 2000). Taken together, the results of these studies demonstrate the complex and dynamic relationship between body weight and calorie intake. This relationship seems to be influenced by diet type and the age at which measurements are taken, potentially among other factors. Indeed, a longer diet administration protocol or beginning diet administration in adulthood may aid in elucidating the effects of different diet types on females, as males in this study were gaining more weight and were more affected by the diet administration protocol than females.

Our results indicate important interactions between sex and diet on plasma leptin levels. First, while diet had no effect on plasma leptin in females at the time it was measured,

males in the SD group had lower leptin levels than males in the HCD and WD groups. Second, while males had higher leptin levels than females, the interaction revealed that this was only the case for males in the HCD and WD groups. A prior study using male rodents only reported elevated leptin levels after 12-week HCD exposure (74% kcal carbohydrate; compared to an SD; J. A. S. Gomes et al., 2020), whereas another group reported increased leptin resistance after nine-week high-fructose diet exposure (compared to an SD; Bursać et al., 2014). Using both sexes, R. B. S. Harris et al. (2003) showed that exposure to a five-week WD (45% kcal fat, 35% kcal carbohydrates; compared to an HCD control) induced leptin resistance in male mice, whereas females remained leptin responsive. Further to this finding, L.-L. Hwang et al. (2010) reported that male C57BL/6J mice administered an HFD (compared to an SD) are more susceptible to weight gain and increases in metabolic hormones (e.g., leptin, insulin) compared to females. Moreover, male rats fed the same WD as in the present work show increased leptin levels and caloric intake compared to those fed a control diet (i.e., the HCD in this work); an effect that was not observed among females (Myles, O'Leary, Smith, et al., 2020). Previous work has shown that male rodents are more susceptible to weight gain after HFD exposure; a finding that may be related to the protective effects of ovarian hormones, such as estradiol in females (Hong et al., 2009; Panetta et al., 2017; Stubbins et al., 2012). These studies and the present work suggest that HFD-induced leptin resistance is occurring more severely, more rapidly, or is more dependent on developmental age in males compared to females and that both sexes are important to study. Furthermore, future comparative studies on diet exposure and sex differences would benefit from including additional measures of metabolic dysfunction (e.g., blood glucose levels, fat pad weights, insulin resistance), as it is evident from our results that metabolic health is intricately related to both sex and diet.

In contrast to our work that did not reveal an effect of diet on CA3 BDNF levels, N. Yamada et al. (2011) showed that levels of BDNF in whole hippocampi were significantly decreased in male mice administered an HFD (16-week administration) compared to an HCD control. Furthermore, Molteni et al. (2002) showed that BDNF levels in whole hippocampi were reduced when rats were administered a 40% fat WD for two months, although only female Fischer 344 rats were used. Our results showed that males had higher hippocampal CA3 BDNF compared to females. Previous work has shown that BDNF in

the CA3 region varies by sex and exposure to stress (Franklin & Perrot-Sinal, 2006); however, it was found that after one-hour restraint stress, male Sprague–Dawley rats had lower CA3 BDNF relative to females (Franklin & Perrot-Sinal, 2006). In another study in which chronic unpredictable stress and short-term tail shock stress were compared between male and female Sprague–Dawley rats, it was found that shock stress decreased serum levels of BDNF only among female rats (Weisbrod et al., 2019). Despite the methodological differences among these studies, there appear to be sex differences in levels of hippocampal BDNF, which warrants further investigation.

The relationships among hippocampal BDNF levels and weight and calorie intake are complex (e.g., Ieraci et al., 2020), and sex-specific research examining hippocampal BDNF (e.g., Franklin & Perrot-Sinal, 2006; Weisbrod et al., 2019) as a metabolic outcome is limited. While BDNF is often associated with cognitive processes (e.g., learning, memory), or mental health (e.g., depression risk), it is also important for maintaining healthy appetite control and body weight (i.e., it is an anorexigenic factor; Vanevski & Xu, 2013). For instance, in human plasma samples, Pillai et al. (2012) found that females had lower BDNF levels compared to males and that body weight and BDNF were only correlated in females. The work previously discussed by Panetta and colleagues (2017) reported more observed anxiety and reduced metabolic health in male rats compared to female rats exposed to 60% HFD. Furthermore, there was evidence of leptin resistance in male rats via downregulation of the *LepR* gene with no change in *Bdnf* expression, whereas female rats showed upregulation of *Bdnf* and no change in *LepR* (Panetta et al., 2017). Interestingly, our study provides some additional support for a sex-specific relationship between metabolic proteins and metabolic health, as the association between hippocampal BDNF protein levels and weight gain was significant in female rats only. Due to the CA3 region of the hippocampus being a crucial location for both leptin and BDNF gene and protein expression (Conner et al., 1997; Cortés-Álvarez et al., 2022; C. Li et al., 2021; Molteni et al., 2002), future work could measure levels of both proteins in this CA3 or other hippocampal regions specifically. Future work may also benefit from including a non-stressed control group as interactions with sex or diet on stress exposure (Aslani et al., 2015; Franklin & Perrot-Sinal, 2006; Weisbrod et al., 2019). Further, studying changes in BDNF and leptin levels following different types of acute or chronic stressors, at different

time points after stress exposure concludes, and with additional food deprivation after behavioural testing would help to elucidate the effects of diet and sex on neurobiological responses to stress.

Strong positive correlations were also found among weight gain, caloric intake, and leptin and BDNF levels overall and in many subsets, with specific correlations with CA3 BDNF appearing based on which subset was being analyzed. Furthermore, certain key behavioural variables and metabolic measures were found to be significantly correlated depending on experimental group (Figure 2.7). Relationships among these variables have been alluded to or directly measured in previous work (e.g., S. Lin et al., 2000; Panetta et al., 2017; Scarpace et al., 2005). Further, Scarpace et al. (2005) demonstrated that inducing leptin resistance via intracerebroventricular injection in rats (male F344xBN) resulted in increased energy intake, weight gain, and fat accumulation when rats were given an HFD, suggesting that leptin resistance caused obesity in their rats. Furthermore, in adult C57BL/6J male mice, subcutaneous leptin injection decreased food intake in normal-fed mice, and not HFD-fed mice (N. Yamada et al., 2011), showing that mice given the HFD were already resistant to the effects of leptin. Although plasma leptin levels and CA3 region BDNF levels were not significantly correlated in our study, due to the design of their study, N. Yamada et al. (2011) were able to show that leptin administration increased hippocampal BDNF levels only in mice fed their control diet, suggesting that the BDNF levels of mice in a leptin-resistant state were not changeable (N. Yamada et al., 2011). Thus, leptin seems to be instrumental in the relationship between physiological hormone or neurotrophin levels (e.g., BDNF) and both obesity-related and mental health outcomes. These effects seem to be limited as to whether the organism is responsive to leptin at the time of measurement.

2.6 Conclusions

This study demonstrates that there are important sex differences in the presentation of anxiety-related and defensive behaviours, along with physiological variables linked to metabolism after diet exposure. These findings should continue to be elucidated mechanistically (e.g., by administering leptin to show a resistant state) in future studies.

While this work does add to the body of research showing that male rodents may be more physiologically and psychologically susceptible to the effects of a WD than females (e.g., Hong et al., 2009; Myles, O'Leary, Smith, et al., 2020; Panetta et al., 2017; Stubbins et al., 2012), the relationship between sex and health outcomes is more complex than it appears. Recent work suggests that female rodents are also susceptible to the metabolic consequences of poor diet but are protected for longer than males and that there are rodent species and strain differences to consider (Maric et al., 2022). Taken altogether, our findings demonstrate that, when compared to an SD, an HCD might not be an appropriate control, as it may lead to detrimental metabolic health effects, similar to those produced by a WD. However, the seemingly improved metabolic state in the rats fed the SD is challenging to characterize as any number of diet components (some of which are proprietary) could be affecting metabolic health on their own or in combination (e.g., fibre, certain contaminants; see Pellizzon & Ricci, 2020 for a review). Even looking beyond broad macronutrient (i.e., protein, carbohydrate, fat) breakdowns reported among different studies, the specific micronutrients, and even non-nutrients (e.g., phytoestrogens, heavy-metals, contaminants in non-purified diets) in rodent diets may have striking effects on findings (Pellizzon & Ricci, 2020).

Characterizing the potential mechanisms of anxiety and obesity development in response to specific diet exposure is pertinent as harmful changes in psychological and physiological variables have implications for overall health (e.g., increase risk of cardiovascular disease, other psychological disorders such as depression; Baker et al., 2017; Chu et al., 2018). Here, we aimed to highlight that SDs and HCDs are two distinct types of control diets that are one of many factors in research that can affect the comparability and reproducibility of studies in this area. Undeniably, the precise dietary composition of control diets is a crucial consideration when studying the effects of a WD on anxiety and making comparisons between studies, as has been previously stated (Pellizzon & Ricci, 2018, 2020; Warden & Fisler, 2008) and directly studied (De la Fuente-Reynoso et al., 2022; Eudave et al., 2018; Gaur et al., 2022; Lang et al., 2019; Sasidharan et al., 2013). Our results suggest that an SD might be more ecologically relevant or generalizable to human outcomes when attempting to control for the effects of an unhealthy WD; however, standard diets are not without their issues. More work is needed to characterize what an ideal control diet might

comprise and how this composition may need to change depending on the specific goals of each study. We recommend that researchers strongly contemplate the type of control diet used in their work, both when interpreting their results and when making comparisons between studies.

2.7 Declaration of Competing Interest

Since 2018, EMM and MEO (with TSP) have been awarded two Mitacs Accelerate Fellowships (IT11380/IT15513 and IT11379/IT15514, respectively). These Mitacs Accelerate Fellowships rely on financial support and in-kind contributions from the Rosell® Institute for Microbiome and Probiotics (the research group of Lallemand Health Solutions Inc.; RIMaP). As well, the Natural Sciences and Engineering Research Council (NSERC) of Canada Engage and Engage Plus (523896-18 and EGP2537192-18, respectively) awards to TSP were also possible because of collaboration and financial support from the RIMaP. The RIMaP and Lallemand Health Solutions Inc. were not involved in data analyses or manuscript preparation. Additional financial support was given to EMM and MEO as Masters and PhD stipends from the Department of Psychology & Neuroscience and the Faculty of Graduate Studies at Dalhousie University, along with Master's and PhD level Nova Scotia Graduate Scholarships. EMM was also awarded a Master's Level NSERC CGS-M award at the time of this work and EMM and MEO have since been awarded PhD Level NSERC CGS-D and PGS-D awards, respectively. No other conflicts of interest are reported.

2.8 Funding

This project was directly supported by two Mitacs Accelerate grants (IT11380 and IT15513) awarded to TSP and EMM, an NSERC of Canada Engage grant (523896-18) awarded to TSP, and an NSERC Engage Plus grant (EGP2537192-18) awarded to TSP, with industry collaboration as described above.

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2.10 CRediT Authorship Contribution Statement

EMM: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft, visualization. **SIH**: conceptualization, formal analysis, investigation, data curation, writing – original draft/review & editing. **SNA**: formal analysis, investigation, data curation, writing – original draft/review & editing. **IDR**: investigation, formal analysis, writing – review & editing. **MEO**: methodology, investigation, writing – review & editing. **TSP**: conceptualization, methodology, formal analysis, resources, writing – original draft/review & editing, supervision, project administration, funding acquisition.

CHAPTER 3 *LACTOBACILLUS HELVETICUS* R0052 AND *BIFIDOBACTERIUM LONGUM* R0175 ADMINISTRATION TO LONG–EVANS RATS HAS INTERACTIVE EFFECTS WITH SEX AND DIET ON ANXIETY-RELATED FEEDING BEHAVIOURS AND SPECIFIC ENDOCRINE OUTCOMES

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In preparation

Keywords: Probiotics; feeding behaviours; sex differences; metabolic hormones; gene expression; cytokines

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Highlights

- Eighty rats of both sexes were given probiotic or placebo, and a specific diet.
- In males, the probiotic interacted with Western diet to yield lower calorie intake.
- Males given standard diet and probiotic had lower anxiety-related behaviours.
- Probiotic rats were lower in various cytokines and higher in NPY than placebo rats.
- Plasma ghrelin was highest, and leptin was lowest in females given standard diet.

3.2 Abstract

Anxiety symptomatology and metabolic functioning are intricately related, with associated mechanisms yet to be fully expounded. Nutritional manipulations, such as probiotic and diet, have been shown to impact health and disease development distinctly in each sex. This work aimed to characterize specific anxiety- and feeding-related behavioural outcomes, complemented by an analysis of molecular alterations in metabolic- and stress-related markers. A total of 80 post-weaning male and female Long–Evans rats were administered the CEREBIOME® probiotic (or placebo) and Western diet (WD; or standard diet, SD) from weaning until sacrifice in adulthood (i.e., ten rats in each of the eight experimental groups). Behaviourally, relative to SD animals, WD animals gained more weight, had higher leptin, consumed more calories during behavioural testing, and lost less weight following a 24-hour fast period. Additionally, compared to their placebo-treated counterparts, rats given probiotic did not have the expected increased calorie intake when fed the WD. As well, probiotic males given SD spent more time in the center of the open field apparatus during testing compared to placebo males given SD. Molecularly, these results are complimented by overall increased hypothalamic cytokine protein levels and lower adrenal neuropeptide Y gene expression in placebo rats compared to probiotic rats. Furthermore, clear sex-specific interactions with diet were evident, with plasma leptin highest in WD males and WD females, plasma ghrelin highest in SD females, and adrenal glucocorticoid receptor gene expression highest in females. These results highlight sex-specific physiological and behavioural effects of both probiotic and WD administration.

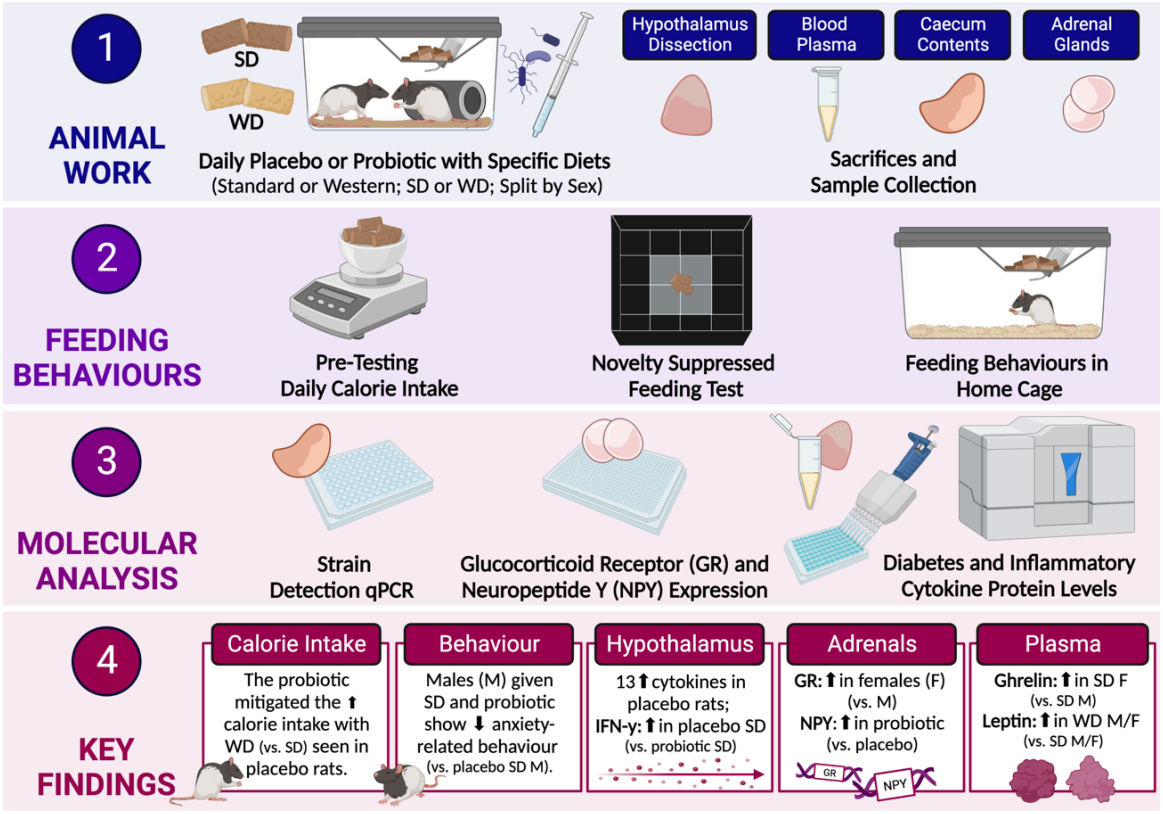


Figure 3.1 Graphical abstract depicting the research project, research timeline, and key findings.

3.2 Introduction

Anxiety disorders and obesity are both common and chronic conditions with associated environmental and genetic risks (Sarma et al., 2021; Walter et al., 2016). Recently, it has been estimated that 12% of the global population is in the 'obese' BMI category (GBD 2015 Obesity Collaborators, 2017), and the worldwide prevalence for any anxiety disorder is estimated at 7.3% (Baxter et al., 2013). Further, human data suggest that both conditions seem to affect females more than males (Baxter et al., 2013; Butnoriene et al., 2015; GBD 2015 Obesity Collaborators, 2017). Importantly, the risk of developing both anxiety and obesity is increased by the presence of the other (Avila et al., 2015; De Hert et al., 2011; Rajan & Menon, 2017). For instance, in a US adult sample, Simon et al. (2006) report that the lifetime prevalence of anxiety disorders (i.e., generalized anxiety disorder, panic disorder with or without agoraphobia) is significantly higher in people with a body mass index (BMI) in the 'obese' category (i.e., above 30 kg/m^2) than people with a BMI below 30 kg/m^2.

It is well established that stress affects food intake bidirectionally (i.e., increases or decreases) depending on factors such as the type or duration of stressor, the type of model organism, or specific characteristics of the subjects or participants (e.g., age, sex, gender, baseline metabolic state, comorbid conditions; Oliver & Wardle, 1999; Razzoli & Bartolomucci, 2016; Torres & Nowson, 2007). As well, psychological distress has been linked to increased palatable or unhealthy food choices (Grieger et al., 2022; D. Hill et al., 2022; M. J. Morris et al., 2015). The presence of metabolic dysfunction or obesity can result in physical (e.g., fatigue, pain) and psycho-social symptoms (e.g., low self-esteem, reduced quality of life) that can further exacerbate anxiety (de Wit et al., 2022; Xiaona Liu et al., 2020; Sarwer & Polonsky, 2016). Relatedly, anxiety symptoms can compound and put individuals at a higher risk of metabolic health complications and disease development (Rofey et al., 2009; G. Zhao et al., 2009). Thus, anxiety and obesity are intricately and bidirectionality related, and the scope of this relationship is heavily routed in individual differences in the physical and psychological response to both conditions (reviewed by Fulton et al., 2022).

Metabolic hormones such as leptin and ghrelin are key anxiogenic and orexigenic (respectively) peptide hormones that are connected to the orexigenic peptide, neuropeptide Y (NPY). Schwartz et al. (1996) showed that injections of leptin to adult male Long–Evans rats resulted in decreased levels of NPY mRNA in the arcuate nucleus (ARC) of the hypothalamus and increased corticotrophin-releasing factor (CRF) mRNA in the paraventricular nucleus (PVN) of the hypothalamus compared to control injections. In the PVN, leptin binds to receptors to stimulate anorexigenic proopiomelanocortin (POMC) neurons and inhibit orexigenic neurons like NPY (Cowley et al., 2001), which has implications for food intake and psychological symptomatology. In contrast, with respect to food intake, ghrelin binds to and stimulates NPY neurons in the ARC and is expressed directly in the PVN (Cowley et al., 2003). Interestingly, glucocorticoid receptor (GR) expression and the inflammatory response seem to be intricately related to appetite control and associated hormones. For instance, an excess of circulating glucocorticoids has been linked to leptin resistance, and ghrelin and NPY might be key hormone modulators in hypothalamic-pituitary-adrenal (HPA) axis responding (reviewed by Kuckuck et al., 2022). As well, male C57BL/6J mice exposed to water immersion stress (three days) had decreased food intake, nesting behaviour, increased IL-6, decreased plasma leptin, and increased plasma ghrelin (C. Yamada et al., 2018).

Differences in gut microbiota composition, along with associated genes (gut microbiome) and metabolites (gut metabolome), are important considerations when studying the relationship between risk and development of anxiety and obesity. The composition of the gut microbiota (e.g., specific taxa presence, low diversity), along with environmental factors that induce dysbiosis (i.e., variations from what is optimal; Lynch & Pedersen, 2016) have been linked to both metabolic dysfunction (Arora et al., 2021; Bäckhed et al., 2004; Dabke et al., 2019; M. Sharma et al., 2020; Trasande et al., 2013; Turnbaugh et al., 2006) and mental health problems (Clapp et al., 2017; Heijtz et al., 2011; Suganya & Koo, 2020). More specifically, the gut microbiota can affect energy balance and metabolism in humans (Turnbaugh, Hamady, et al., 2009), and low diversity of the bacterial species in the gut microbiota is associated with increased risk of poor metabolic health (e.g., adiposity, insulin resistance; Le Chatelier et al., 2013). For instance, reduced abundance of beneficial bacterial genera (*Bifidobacterium* and *Lactobacilli*) has been reported in infant

rhesus monkeys exposed to prenatal stress (acoustic startle stress to pregnant mothers; Bailey et al., 2004). Moreover, in humans with anxiety disorders, lower microbial diversity and increased prevalence of harmful or pathogenic bacteria genera (e.g., *Bacteroides*; *Escherichia*) have been reported (Jiang et al., 2018; C. A. Simpson et al., 2020).

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (C. Hill et al., 2014, p. 507). Important to this definition is that a product can only be considered a probiotic if the strains (e.g., of bacteria, yeast) in question are provided at a specific dosage and have been studied for a specific purpose (i.e., excluding vague claims without strains and dosages, fermented foods, and fecal transplants; C. Hill et al., 2014). Probiotics have strain-specific effects, and not all probiotics are studied for broad-spectrum use (Gareau et al., 2010). Overall, the goal of administering individual strains of probiotics is to promote specific health outcomes (e.g., reducing depressive symptoms; C. J. K. Wallace & Milev, 2017) and reduce disease risk or symptoms (e.g., bloating in irritable bowel syndrome; Yan Zhang et al., 2016). In contrast, administering unhealthy diets in rodents (e.g., a Western diet, WD) has the goal of modelling unhealthy diet exposure in humans quickly and in a controlled environment (Bastías-Pérez et al., 2020). More specifically, previous animal research has documented beneficial changes in body weight (An et al., 2011; Holowacz et al., 2015), metabolic hormones (Al-muzafar & Amin, 2017; Holowacz et al., 2015), inflammatory cytokines (Desbonnet et al., 2010; N. Li et al., 2018), and anxiety-related behaviours (Ait-Belgnaoui et al., 2012, 2018), with the administration of *Lactobacillus*- and *Bifidobacterium*-containing probiotics. Alternatively, administration of various high-fat diets (HFDs) and WDs to rodents has been reported to worsen metabolic and inflammatory parameters (Cani et al., 2007; De Souza et al., 2005), along with affecting anxiety-related behaviours inconsistently (i.e., increase or decrease depending on the study; e.g., increased anxiety in Dutheil et al., 2016; reduced anxiety in McNeilly et al., 2015), with differential findings in each sex (Bridgewater et al., 2017; Maniam & Morris, 2010; Soulis et al., 2007).

The objective of the present work was to study psychological and physiological outcomes of CEREBIOME® administration (i.e., combination of 90% *Lactobacillus*, *L., helveticus* R0052 and 10% *Bifidobacterium*, *B.*, *longum* R0175; previously known as Probio'Stick®)

in combination with unhealthy WD. Previous rodent work has reported on alterations in anxiety-related behaviours, metabolic parameters, and cytokine levels with probiotic (e.g., CEREBIOME®) treatment and WD administration, but literature that examines the interactive effects of both nutritional factors, especially in each sex, is severely lacking. Using Long–Evans rats, we examined the impact of probiotic (or placebo) treatment, WD (or standard diet, SD) administration, and sex on weight and food intake measures, anxiety-related and feeding behaviours, metabolic hormone levels in plasma, gene expression of adrenal NPY and GR and, and cytokine levels in hypothalamus tissue. Broadly, we hypothesized that CEREBIOME® treatment would improve metabolic, inflammatory, and anxiety-related outcomes, and counteract some negative impacts of WD administration (Ait-Belgnaoui et al., 2012, 2018; Al-muzafar & Amin, 2017; Avolio et al., 2019; Myles, O'Leary, Romkey, et al., 2020). Previous research also suggests that male rats are quicker to show poorer metabolic health in response to WD administration and display more anxiety-related behaviours during testing (Hong et al., 2009; Myles et al., 2023; Myles, O'Leary, Smith, et al., 2020; Panetta et al., 2017; J. Simpson et al., 2012). Thus, we hypothesized that the male rats in this study would display more indicators of poorer metabolic health and psychological distress than females.

3.3 Materials and Methods

3.3.1 Animals and Housing

This study and its procedures (Figure 3.2) were approved by the Dalhousie University Committee on Laboratory Animals (protocol #20-131), following both the Canadian Council on Animal Care and ARRIVE guidelines. Long–Evans hooded rats were ordered for breeding (specific pathogen-free, viral antibody-free; Charles River Laboratories, Raleigh, NC, USA) and quarantined for 14 days upon arrival to the animal housing facility at Dalhousie University's Life Sciences Centre. The side-by-side, identical colony rooms were on a reversed 12:12 hour light-dark cycle (lights off at 10:00 h; 11:00 after daylight savings time began in March 2021), and temperature controlled at 20 ± 2 °C. Rats were housed in standard polypropylene cages (47.0 cm × 24.0 cm × 20.5 cm) with wire hoppers

(stainless steel), microisolator lids, softwood bedding (Shaw Resources, Shubenacadie, NS, Canada), and a black PVC tube (12 cm long, 4 cm radius). Breeders were given free access to food (Laboratory Rodent Diet #5001, LabDiet®, St. Louis, MO, USA) and water (double-filtered municipal tap water, glass bottles with stainless steel sippers).

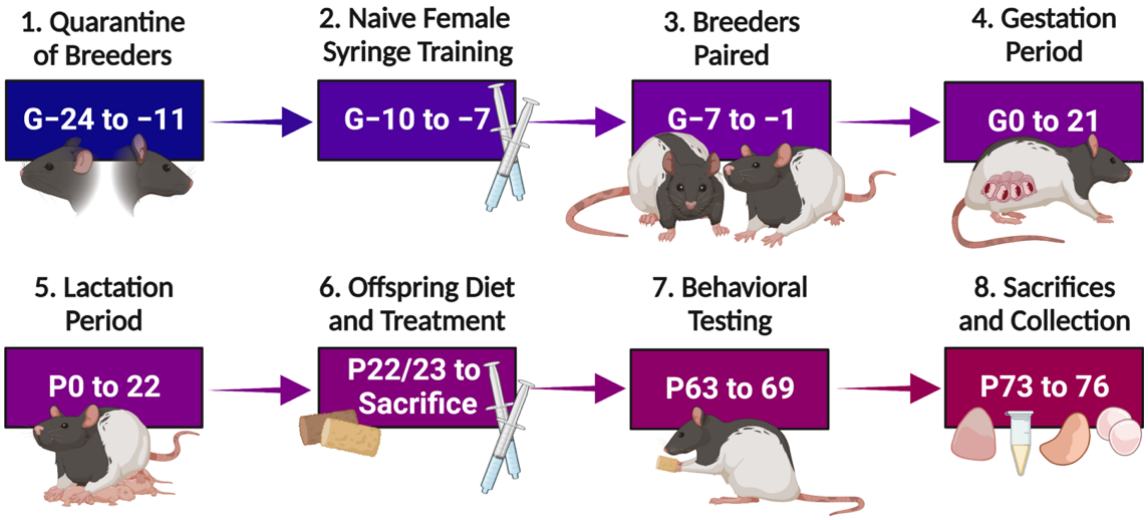


Figure 3.2 Experimental timeline from breeder quarantine to sacrifice of offspring (120 days total). G = gestational day (−24 – 21), P = postnatal day (0 – 76). Created with BioRender.com.

3.3.2 Breeding Protocol

Breeder males (200 – 225g; *N* = 20) were paired with breeder females (225 – 250g; *N* = 20) for a period of seven days, after which, females were assumed to be pregnant. Post-breeding, males were single-housed and given wooden blocks (untreated poplar wood, Home Depot, Halifax, NS) and enviro-dri® (natural brown, Shepherd Specialty Papers, Watertown, TN, USA) for enrichment. Post-bred females were pair-housed until the earliest estimated gestational day 17 (G17) to minimize added stress and resulting offspring effects that can occur with single-housing (Lopes et al., 2022). After single-housing females, litters began to be born six to nine days thereafter (i.e., four litters per day over four days; 16 litters total). During the pregnancy and lactation periods, all females were given enviro-dri® (Shepherd Specialty Papers, USA) for enrichment. Weight of all females was monitored weekly throughout the pregnancy and lactation periods.

3.3.3 Probiotic Administration to Female Breeders

Female breeders were pseudo-randomly assigned to the placebo or probiotic solution upon their arrival to the facility by the animal care department by placing them in either the placebo ($N = 10$) or probiotic ($N = 10$) colony room. After the 14-day acclimatization and prior to breeding, all females began a four-day training session on receiving probiotic or placebo solutions via voluntary syringe feeding. This protocol was developed by Tillmann and Wegener (2018), and it has been previously implemented by our group in Myles, O'Leary, Romkey, et al. (2020) and Myles, O'Leary, Smith, et al. (2020). The probiotic (i.e., CEREBIOME®, previously known as Probio'Stick®, Lallemand Health Solutions Inc.) is a combination of 90% *Lactobacillus helveticus* R0052 and 10% *Bifidobacterium longum* R0175, with excipients (i.e., malic acid, xylitol, and maltodextrin). The placebo powder contains only the excipients. Briefly, the syringe feeding protocol training involves offering the syringe (cat. #309659, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) through the wire cage bars and hand-feeding rats, if needed, until rats begin to approach the syringe voluntarily without any hand-feeding (see Myles, O'Leary, Romkey, et al., 2020; Myles, O'Leary, Smith, et al., 2020; Tillmann & Wegener, 2018).

Because the probiotic and placebo solutions are slightly sweet with the addition of xylitol and maltodextrin, rats quickly learn to approach the syringe and feed. Training the females on the syringe feeding protocol for four days prior to breeding is sufficiently long enough for them to voluntarily feed from the syringe after the breeding period. Administering the probiotic and placebo solutions during the breeding period is not ideal, as males will attempt to overpower females in their quest for the palatable syringe contents, which can result in unnecessary stress for both breeders. After the breeding period (i.e., a seven-day pause in feeding), the pair-housed females all voluntarily approached the syringe and drank the probiotic or placebo solution through the wire hopper bars without needing to hand-feed or remove cages from the cage racks. Subsequently, all females were administered probiotic or placebo solutions daily during the gestational and lactation periods, until their day of sacrifice after offspring weaning.

Each day, the probiotic or placebo solutions were prepared fresh by dissolving a specific

weight (g) in reverse osmosis water (0.5 mL administered per rat per day). The probiotic dose was 5×10^8 colony-forming units (CFUs) per day (or 0.01 g of powder per 0.5 mL of water). The placebo rats were also given 0.01 g of placebo powder in 0.5 mL of reverse osmosis water each day. Both probiotic and placebo solutions were prepared daily following the cross-contamination protocols described in detail by Myles, O'Leary, Romkey, et al. (2020) and kept on ice until administration. The raw powders were kept refrigerated (4 °C ± 4 °C) on different shelves and double-bagged for the duration of the experiment. Daily probiotic and placebo solutions were always administered at the start of the dark phase of the light-dark cycle (i.e., 11:00 h ± 1 h before daylight savings time began; 12:00 h ± 1 h after daylight savings time began). Solutions were administered in each colony room concurrently by four trained experimenters (i.e., two experimenters per day) working on this project. The experimenters were counterbalanced each day of administration to ensure that rats were not exposed to different experimenters as a whole.

3.3.4 Offspring Experimental Groups

In both the placebo and probiotic colony rooms, eight of ten bred females successfully produced litters; however, only litters with six or more pups were used for the present experiment (F. A. Champagne et al., 2003). In the placebo room, offspring from six of the eight litters were used (one litter of two and one litter of four were used in another experiment). In the probiotic room, offspring from seven of the eight litters were used (one litter of five was used in another experiment). The average birth weights were 6.41 g (*SD* = 0.43) in the placebo room and 6.37 g (*SD* = 0.56) in the probiotic room. At weaning (postnatal day, P, 22), 40 offspring from each room were sorted into eight experimental groups (see Figure 3.3), ensuring that no more than two rats per litter were represented in each group. The remaining offspring were used in a separate experiment in our lab. Weaning of offspring was conducted after the administration of probiotic and placebo solutions to dams finished that day, so direct offspring probiotic and placebo treatment began at P23. Post-weaning offspring were housed in same-sex and same-litter pairs as described in Section 3.3.1.

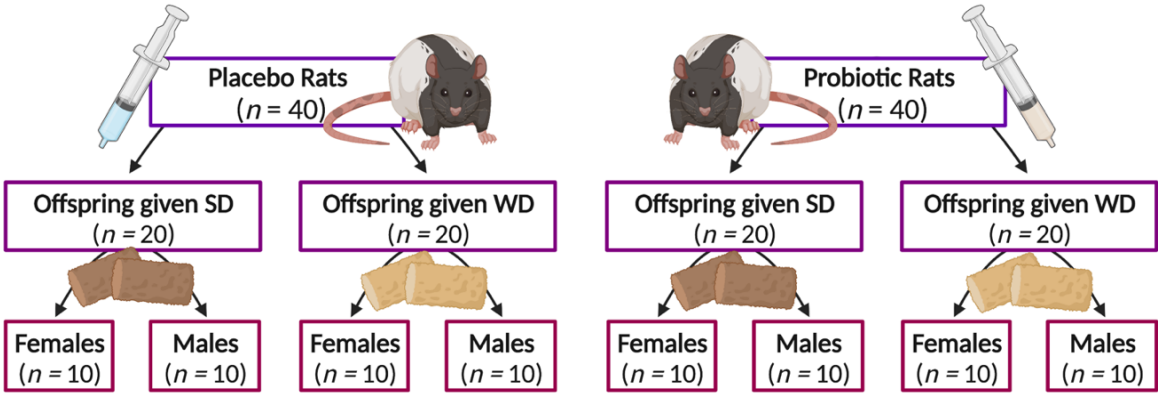


Figure 3.3 Experimental group depiction showing that of the 40 Long–Evans rats that received placebo and the 40 that received probiotic, 20 of each group (10 of each sex) were additionally administered Western diet (WD) or standard diet (SD). Created with BioRender.com.

3.3.5 Diet Administration to Offspring

From weaning (P22) until sacrifice, all rats were fed a specific rat diet (i.e., SD vs. WD) *ad libitum* for a period of at least seven weeks (until sacrifice between age P73 and P76). The WD (cat. #D12079B, Research Diets, New Brunswick, NJ, USA) was 40% fat, 43% carbohydrates, and 17% protein (4.7 kcal/g; see Myles et al., 2023). The SD was a traditional Purina rat lab chow (LabDiet®, cat. #5001; 13% fat, 29% protein, and 58% carbohydrate; 3.4 kcal/g; see Myles et al., 2023). An SD was chosen as per recommendations from Myles et al. (2023) and to compare or contrast present findings to a similarly designed study using a high-carbohydrate control diet (Myles, O'Leary, Smith, et al., 2020).

3.3.6 Probiotic Administration to Offspring

One day post-weaning (P23), the 80 offspring rats were administered probiotic or placebo (see Figure 3.3) until sacrifice (P73 to P76), based on whether their mother was designated as a probiotic or placebo rat. Administration and dosage protocols were the same as described in Section 3.3.3. As with the dams, voluntary administration of the syringe with the probiotic or placebo requires a training period. Previous experience with newly weaned

rats has shown that they take considerable time to voluntarily syringe feed (27 days maximum in Myles, O'Leary, Smith, et al., 2020), and it was suspected that this might be due to the size of their mouths relative to the size of the 1 mL syringe (cat. #309659, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Thus, in this experiment, we added feeding tips to the 1 mL syringe (Fisherbrand™ Disposable Animal Feeding Needles, cat. #01-208-87, Thermo Fisher Scientific, Whitby, ON, Canada). Since the syringe feeding protocol includes an initial attempt at feeding through the wire cage bars as a baseline, it was noted that some offspring fed immediately from the syringe with a feeding tip with no training required (i.e., did not need hand feeding). Detailed daily notes were taken for each of the 80 offspring to track their progress to voluntary feeding so that all experimenters knew which rats were comfortable and which needed to be handfed. All 80 offspring fed directly from the syringe (with added tip) from the cage rack within seven days. As the offspring got subjectively bigger and more comfortable or eager to feed (e.g., males on the Western diet), the feeding tip was able to be removed progressively. For consistency, offspring who did and did not need the added feeding tip were noted in a colony room-specific lab notebook, which was reviewed daily prior to probiotic or placebo administration.

3.3.7 Food Intake and Weight Measures

All offspring were weighed at weaning, and weekly weights were further recorded at the exact ages of P31, P38, P45, P52, and P59 (prior to any behavioural testing). Between ages P50 and P55, available food was measured daily (i.e., every 24 h ± 15 mins) to calculate the change in food intake (g) over a five-day period. As commonly calculated, the average change in food weight each day was divided by the number of rats per cage (i.e., by two) on the assumption that cages were splitting calories 50:50 (e.g., in Maniam & Morris, 2010). Then, the average daily food intake per rat was converted to average daily calorie intake (absolute kcal) by multiplying the kcal/g of that specific diet by the average daily weight (g) of food eaten. However, a relative kcal value was also calculated for each rat based on their specific P59 weight to help account for the fact that heavier rats would be contributing more to the cage's calorie intake (described in Myles et al., 2023).

3.3.8 Novelty-Suppressed Feeding Task

Behavioural testing occurred over a period of ten days (ages P63 – P69), with eight rats being tested per day following a strictly scheduled checklist to ensure that timing of food deprivation and type of diet administration was accurate. Prior to testing, all rats (by cage) were food-deprived and allowed free access to water. At the same time as food deprivation, all rats were weighed so that a 24-hour fasting weight could be reported (24-hour fasting weight for all rats was measured between 24 h 8 min and 25 h 14 min). Testing occurred in the middle of the dark phase of the light-dark cycle to ensure activity level would be sufficient for testing (Roedel et al., 2006) and to keep timing consistent between animals (as per Nelson et al., 2021). As well, placebo animals were tested (between 14:00 h and 16:00 h) prior to probiotic animals (between 16:30 h and 18:30 h) due to the risk of cross-contamination from probiotic to placebo animals (e.g., from bedding, cages; described in Myles, O'Leary, Romkey, et al., 2020). Because many daily environmental factors might affect behaviour of rats during testing, testing sessions were counterbalanced with rats from four of the eight experimental groups tested each day (two cage mates per group). Exceptions were made to this default based on rat ages to keep the age range to a minimum.

All 80 rats were tested in a novelty-suppressed feeding task (NSFT; Bodnoff et al., 1988; Gross et al., 2000; Ochoa-Sanchez et al., 2012; Samuels & Hen, 2011) in a novel open field apparatus (80 cm × 80 cm × 35 cm high; black plexiglass; C. Hall & Ballachey, 1932; Figure 3.4) after 24-hours of food deprivation. The exact timing from food deprivation to NSFT testing for all rats was between 24 h 1 min and 24 h 51 min. The floor of the OF apparatus was divided into a grid of 16 equal squares with Fisherbrand^{TM} labelling tape (Thermo Fisher Scientific, Whitby, ON, Canada; center area 4/16 inner squares, 25% of total floor space). Testing was conducted under white light with two 26 W compact fluorescent light fixtures (General Electric, model number f26dbx/841/eco4p; 1440 mean lumens each) mounted to the ceiling 235 cm above the OF apparatus. All rats were transported to one specific nearby testing room (same floor as colony rooms) in a holding cage containing regular bedding. Prior to transport, holding cages were covered by a dark towel specific to the probiotic or placebo colony room, and water was removed. During the 10-minute test, three pre-weighed food pellets of the rat's specific diet (i.e., SD or WD)

were placed in the center in a weighted (unmovable) dish. At the start of the test, all rats were placed in the apparatus in the perimeter area facing the center in an identical manner. The testing apparatus was cleaned with 70% ethanol between trials and before the first trial of the day.

Variables that were live-recorded during the NSFT were initial weight of the three food pellets (in grams, g), time of first feeding bout (> 3 s), total number of complete feeding bouts, and final weight of remaining food pellets. During optimization of the NSFT protocol with Long–Evans rats that were borrowed from another experiment, an attempt was made to affix the food pellets to the feeding dish. Attempts were unsuccessful overall as all measures to keep the pellets affixed (e.g., glue gun, rubber bands) were insufficient in keeping the rats from acquiring and caching them in the perimeter. To work within this limitation, we decided to add measures of perimeter and center pellet contacts (any body part except tail) and pellet carries (usually with the mouth but sometimes with front paws while hopping bipedally) in any region of the apparatus. All NSFT sessions were video-recorded (Sony Handycam® DCR-SR68 video camera), which allowed for later scoring of anxiety-related and defensive behaviours (i.e., line crosses, supported rearing on perimeter, unsupported rearing, latency (s) to enter center, time (s) in center, and center transitions; see Table 3.1). This retrospective scoring was counted or timed with Behavioral Observation Research Interactive Software (BORIS, version 7.10.7; Friard & Gamba, 2016) by two observers (with an equal number of rats by treatment, diet, and sex) but was not blinded as both rat IDs and diet type could be gauged from the videos.

3.3.9 Home Cage Feeding Observation

After concluding the NSFT, rats were returned to colony rooms in holding cages. Their cage mate was placed into a holding cage, and the original home cage was used for a home cage observation. To begin the 5-minute, live-scored home cage observation, 25 – 50 g of pre-weighed food and a water bottle were added to the cage. The initial and final weights of food (g), latency to first feeding bout (600 s maximum), and number of feeding bouts (greater than 3 s) were recorded. Timing of food deprivation until home cage observation ranged from 24 h 1 min and 25 h 6 min.

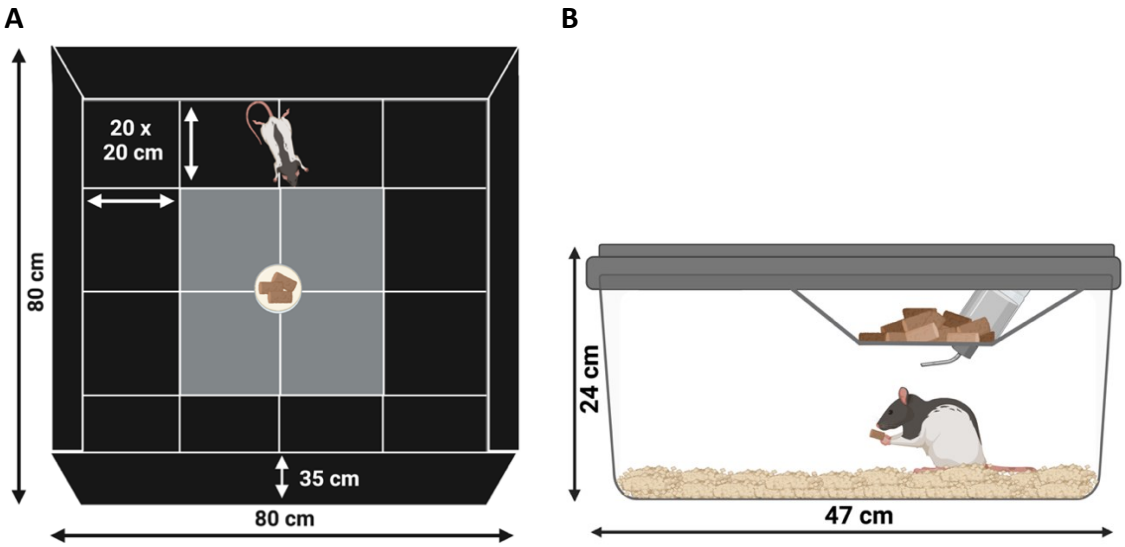


Figure 3.4 **A.** Novel open field apparatus for novelty-suppressed feeding task; and **B.** Standard housing cage (21 cm deep) for home cage observation. Created with Biorender.com.

Table 3.1 Ethogram of anxiety-related variables in the novelty-suppressed feeding task.

Behaviour (Category)	**Description**
Line Crosses (Locomotion)	Frequency measure of all four paws crossing between quadrants in any orientation (Kalueff & Tuohimaa, 2004)
Supported Rearing (Locomotion)	Frequency measure of supported (on perimeter; one or both paws) vertical extensions on hind legs with any return to ground marking the end of the behaviour (Sturman et al., 2018)
Unsupported Rearing (Exploration)	Frequency measure of unassisted (both paws off ground) vertical body extensions on hind legs with any return to ground marking the end of the behaviour (Sturman et al., 2018)
Center Entries (More → Lower Anxiety)	Frequency measure of transitions into the center area with all four paws (Kalueff & Tuohimaa, 2004)
Time in Center (More → Lower Anxiety)	Total time (s) spent (four paws) in the inner four quadrants of the apparatus (Kalueff & Tuohimaa, 2004)
Latency to Enter Center (Less → Lower Anxiety)	Total time (s) taken for all four paws to enter the inner four quadrants of the apparatus (Kalueff & Tuohimaa, 2004)

Note. Unsupported rearing is interpreted as an exploratory behaviour and is interpreted as a lower anxiety indicator only when combined with other measures of lower anxiety.

3.3.10 Tissue and Plasma Collection

Between ages P73 and P76 (over seven days), offspring were sacrificed by intraperitoneal injection of Euthanyl® (sodium pentobarbital, DIN 00141704; 0.3 mL per 100 g of body weight), followed by decapitation by guillotine after confirmation of an absent toe pinch reflex. An equivalent number of probiotic and placebo animals were sacrificed each day, and efforts were made to keep other groups (diet and sex) balanced unless rats were not aged between P73 and P76 on that given day. Immediately after decapitation, 1 mL of whole trunk blood was collected into a pyrogen-free sterile 1.7 mL microtube (cat. #87003-294, VWR, Mississauga, ON, Canada) containing 15 µL of sodium heparin (DIN 02303086, Sandoz Canada Inc., Boucherville QC) and placed on ice. Then, while working on dry ice, one trained experimenter carefully removed the brain from the skull and gross dissected the hypothalamus from a section spanning from plates 27 – 37 (Paxinos & Watson, 1986) into a 1.7 mL microtube (cat. #87003-294, VWR). Simultaneously, a second trained experimenter collected both adrenal glands into a 1.7 mL microtube (cat. #87003-294, VWR) and flash-froze the tube on dry ice. Then, this second experimenter dissected out whole caecum and flash-froze it on dry ice. Each day, plasma was prepared in two centrifugation steps: spinning at 1,000 *g* for 15 mins and collecting the supernatant (plasma), then performing a second spin at 10,000 *g* for 10 mins and collecting the supernatant (plasma) again. The collected hypothalami, adrenals, caeca, and plasma were stored at −80 °C until further analysis (i.e., between five and eight months after collection).

3.3.11 DNA Extraction from Caecum Contents of Offspring

In a biosafety cabinet, 200 – 350 mg of caecum contents were removed from caecum lining using a new sterile disposable swab tip (i.e., swabs were held in reverse utilizing the sterile hard plastic end; cat. #25-3406-HBT, Puritan Medical Products, Guilford, ME, USA). Contents were placed into autoclaved bead beating tubes containing four 3 mm glass beads (cat. #11-312A, Thermo Fisher Scientific). Caeca were placed on dry ice during removal as the lining removal is cleanest when contents are in a frozen state. Placebo caecal contents were processed prior to probiotic to minimize risk of cross-contamination as per the

protocol outlined in Myles, O'Leary, Romkey, et al. (2020). To extract caecum content DNA from samples, the QIAamp® Fast DNA Stool Mini Kit (cat. #51604, QIAGEN) was used following manufacturer's instructions, but with two modifications (MacPherson et al., 2018): 1) two washes with 0.05 M phosphate buffered saline (PBS; HyClone™, cat. #SH3025602, Thermo Fisher Scientific) prior to adding InhibitEX; and 2) adding a 1 mm silica/zirconia bead beating step prior to centrifugation. This bead beading step involved adding 250 – 350 mg of pre-autoclaved silica beads (cat. #11079110Z, BioSpec Products) per bead beating tube (cat. #72.693.005, Sarstedt) and bead beating for three cycles of 4 m/s for 1 min each (MP FastPrep® 24 5G Homogenizer, MP Biomedicals). DNA concentrations were assessed using the NanoDrop™ 8000 Spectrophotometer (cat. #ND-8000-GL, Thermo Fisher Scientific). DNA samples are acceptable as pure DNA if they show a 260/280 ratio of 1.8 – 2.0 (Thermo Fisher Scientific, 2009). Concentrations for the 80 caecum content samples ranged from 69.23 – 1,221 ng/µL of DNA, with 260/280 ratios between 1.84 and 2.00. All DNA samples were normalized to 20 ng/µL from nanodrop values and stored at −20 °C until qPCR for detection of the probiotic strains was conducted.

3.3.12 qPCR Validation: Control Rat Screening

Four control rats who were never exposed to the probiotic strains used in this experiment (i.e., *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) yielded two caecal content samples each (eight samples total). Caecum lining was removed, and caecal content DNA was extracted as described in Section 3.3.11. In duplicate, all caecal content samples (195 – 360 mg each) were first screened with qPCR to confirm the absence of target strain bacteria (i.e., R0052 and R0175). Screening plates included positive control bacterial DNA for each strain, along with no template negative controls (NTCs) for each strain's master mix. *Lacticaseibacillus rhamnosus* R0011 was also included in the validation experiments and eventual strain-detection qPCR because it was a concurrently used probiotic strain (part of Lacifodil ®, along with R0052) in our facility.

For the control rat screening, qPCR was conducted in a 25 µL reaction volume (22.5 µL master mix, 2.5 µL 1 in 5 diluted DNA) on a 96-well plate in duplicate (MicroAmp™ Fast Optical 96-well reaction plate, cat. #4346907, Applied Biosystems™, Thermo Fisher

Scientific), using the CFX96 Touch™ Real-Time PCR Detection System (cat. #1855196, Bio-Rad). Primers (Table 3.2) were reconstituted to 100 µM with nuclease-free water (cat. #W4502-1L, Sigma-Aldrich), and annealing temperatures were 60 °C. Per reaction, master mixes were comprised of 12.5 µL SYBR (5 mL SYBR™ Select Master Mix, cat. #4472908, Applied Biosystems, Thermo Fisher Scientific), 0.075 µL of forward primer (300 nM final concentration; Table 3.2), 0.075 µL of reverse primer (300 nM final concentration; Table 3.2), and 9.85 µL nuclease-free water (cat. #W4502-1L, Sigma-Aldrich). All positive controls were amplified as expected, with no NTC amplification. In the eight control rat DNA samples, no amplification was found for R0052, R0011, or R0175. Since control caecal content samples did not contain R0011, R0052, or R0175 strains, we proceeded with the following spiking experiment to generate strain-specific standard curves for later strain detection qPCR.

Table 3.2 Gene product sizes (base pairs) and sequences (forward and reverse) for bacterial strain-specific primers (R0175, R0052, R0011) used for qPCR, along with the 16S rRNA gene to confirm presence of bacterial DNA.

Strain or Gene	Product Size (bp)	Forward Primer	Reverse Primer
R0175 (Myles, O'Leary, Romkey, et al., 2020)	99	5′ – GTC GCC ACA TTT CAT CGC AA – 3'	5′– GAG AGC TTC GAT TGG CGA AC – 3'
R0052 (Myles, O'Leary, Romkey, et al., 2020)	150	5′ – AGA ATC AAG CAG AGA CTG GCT ACG – 3'	5′– GGA CCG GAT TTG AGT AGA GGT A – 3'
R0011 (Myles, O'Leary, Romkey, et al., 2020)	71	5′ – ACT CCA AAG AGC ATT ACC TCC G – 3′	5′ – TGA ATA TGC CGG ATC TAA GTC CA – 3′
Bacterial 16S (Amann et al., 1990; Hartman et al., 2009)	180	5′ – ACT CCT ACG GGA GGC AGC AGT – 3′	5′ – ATT ACC GCG GCT GCT GGC – 3′

bp = base pair.

3.3.13 qPCR Validation: Spiking Experiment

A spiking experiment, whereby we used a pooled matrix of control caecum DNA that was 'spiked' with the three bacterial strains, was conducted in order to generate standard curves for eventual strain-detection qPCR in experimental rats. Specifically, caecum contents from all control rats were pooled (3,844 mg caecum total) to form a matrix to which to individually spike (add) the strains. The pooled caecum content matrix was divided into 12 tubes (200 – 250 mg of contents per tube), whereby three replicate tubes for each strain (nine total) were spiked with each of the three probiotic strains. Additionally, one tube for each strain served as unspiked negative controls (i.e., contained only pooled caecum contents). Amplification by qPCR of these unspiked controls was used to inform Cq cut-off area in strain-detection qPCR in the experimental samples. DNA from the caecum contents was extracted from each of the 12 tubes as described in Section 3.3.11. DNA concentrations were assessed using the NanoDrop™ 8000 Spectrophotometer (cat. #ND-8000-GL, Thermo Fisher Scientific), and concentrations ranged from 196.03 – 680.94 ng/µL with 260/280 ratios from 1.82 to 1.97.

To prepare for the spiking experiment, R0011 lyophilized probiotic powder (2.46×10^{11} CFU bacteria/g, lot #30VD0469), R0052 lyophilized probiotic powder (3.36×10^{11} CFU bacteria/g, lot #JF5002), and R0175 lyophilized probiotic powder (3.85×10^{11} CFU bacteria/g, lot #U120201807) were used. As 10^{11} CFU/g of bacteria is too much DNA for our qPCR protocol with these strain-specific primers (i.e., previous optimization has shown that amplification is inhibited), the specific spiking solutions to be added to each spiked sample were prepared at a 1 in 10 dilution using 9 mL of HyClone™ PBS (cat. #SH3025602, Thermo Fisher Scientific) and 1 g of lyophilized bacterial strain powder. Then, to determine the volume of strain-specific spiking solutions to add to the nine samples to normalize to 1×10^{10} CFU, the formula C1V1 = C2V2 was used. Specifically, for the three R0011 caecum content tubes, 407 µL of R0011-specific spiking solution was added, leaving the fourth tube untouched as the unspiked control sample. Likewise, 298 µL of R0052-specific spiking solution and 260 µL of R0175-specific spiking solution were added to the appropriate tubes, with two additional tubes untouched as unspiked samples.

qPCR was conducted in a 25 µL reaction volume (22.5 µL master mix, 2.5 µL 1 in 10 serially diluted DNA) on a 96-well plate (MicroAmp™ Fast Optical 96-well reaction plate, cat. #4346907, Applied Biosystems™, Thermo Fisher Scientific), using the CFX96 Touch™ Real-Time PCR Detection System (cat. # 1855196, Bio-Rad). Cycling conditions are described in Table 3.3, with annealing temperatures at 60 °C. All spiked samples, unspiked samples, and no template controls (i.e., master mixes with specific primers only) were added in duplicate. Primers (Table 3.2) were reconstituted to 100 µM with nuclease-free water (cat. #W4502-1L, Sigma-Aldrich). Per reaction, master mixes (22.5 µL total volume per reaction) were comprised of 12.5 µL SYBR (5 mL SYBR™ Select Master Mix, cat. #4472908, Applied Biosystems, Thermo Fisher Scientific), 0.075 µL of forward primer (300 nM final concentration, Table 3.2), 0.075 µL of reverse primer (300 nM final concentration, Table 3.2), and 9.85 µL nuclease-free water (cat. #W4502-1L, Sigma-Aldrich).

For each strain, one of the three spiked samples was loaded as 10-fold serial dilutions (10^{10} to 10^{3}) to calculate PCR efficiency. PCR Efficiency for R0011 was 95.0%, efficiency for R0052 was 93.4%, and efficiency for R0175 was 90.1%. The other two spiked samples were added from 10^{10} to 10^{8}. From the results of the spiking experiment, the 'best' spiked sample of the three from each strain was used to generate standard curves for the qPCR plates in Section 3.3.14. The decision on which spiked sample was best was based on which of the three test spiking mixes had the tightest Cq values between duplicates and which had the earliest Cq amplification (i.e., spiked mix number 3 was chosen for all strains).

3.3.14 Detection qPCR for 16S and Probiotic Strains

Using the ep*Motion*® *5075t* liquid handling robot (cat. #5075006022, Eppendorf), caecum content DNA that was extracted from mother rats ($N = 16$) and offspring rats ($N = 80$; see Section 3.3.11) was normalized to 20 ng/µL. All DNA was diluted 1 in 5 with nuclease-free water (cat. #W4502-1L, Sigma-Aldrich) prior to qPCR analysis. On top of the plates that would be prepared for strain detection, an additional plate was prepared to confirm presence of bacterial DNA (16S gene). The liquid handling robot was programmed to prepare two sets (i.e., one set for mother rat caecum contents, one set for offspring rat

caecum contents) of four (i.e., one each for R0011, R0052, R0175, and 16S) 384-well plates (eight plates total; clear shell/white well, cat. #HSP3805, Bio-Rad). Plates were prepared in a 10 µL reaction volume, with 1 µL diluted and normalized DNA and 9 µL master mix. All primers (Table 3.2) were reconstituted to 100 µM with nuclease-free water (cat. #W4502-1L, Sigma-Aldrich). Per reaction, master mixes for each primer set (i.e., forward and reverse primers for 16S, R0011, R0052, R0175) were comprised of forward and reverse primers (0.03 µL each, 300 nM final concentration), SYBR (5 µL; 5 mL SYBR™ Select Master Mix, cat. #4472908, Applied Biosystems, Thermo Fisher Scientific), and nuclease-free water (3.94 µL; cat. #W4502-1L, Sigma-Aldrich).

For the six strain detection plates (i.e., for the *Lacticaseibacillus rhamnosus* R0011 plate, *Lactobacillus helveticus* R0052 plate, and *Bifidobacterium Longum* R0175 plate), sample DNA from rat caecum contents (80 offspring, 16 mothers) was added in triplicate. Standard curves were added in duplicate from 10^{10} to 10^{4}, along with no template controls and unspiked DNA (as negative controls) in duplicate. To confirm presence of bacterial DNA, two 384-well plates (clear shell/white well, cat. #HSP3805, Bio-Rad) for 16S detection (no standard curve) were also prepared in triplicate, with the aforementioned controls in duplicate. All plates were sealed with sealing film (Bio-Rad, Microseal B PCR Plate Sealing Film, cat #MSB1001), vortexed, and quick spun. All eight 384-well plates (clear shell/white well, cat. #HSP3805, Bio-Rad) were analyzed with the CFX384 Touch™ Real-Time PCR Detection System (cat. #1855485, Bio-Rad), with the cycling conditions described in Table 3.3, and annealing temperatures of 60 °C. For all eight plates, melt curve analysis was performed to verify the amplification specificity of primers. Melting temperatures were 84.00 – 84.50 °C for all samples, controls, and standards on the R0175 plate, 77.50 °C for R0052, 77.00 °C for R0011, and 84.00 – 86.00 °C for 16S. For the three 384-well plates with standard curves for the 16 mother rats, PCR efficiencies were 95.5% (R^2 = 0.999) for the R0175 plate, 80.3% (R^2 = .989) for the R0052 plate, and 96.7% (R^2 = 0.999) for the R0011 plate. For the three 384-well plates with standard curves for the 80 offspring, PCR efficiencies were 100.2% (R^2 = 0.999) for the R0175 plate, 81.6% (R^2 = 0.988) for the R0052 plate, and 97.6% (R^2 = 0.999) for the R0011 plate.

Table 3.3 Cycling conditions broken into four stages for all qPCR or RT-qPCR in this study, including number of rounds, temperature(s) at each stage, and duration.

Stage	Rounds	Temperature	Duration
1. Predenaturation	1	50 °C	2 mins
2. Primer extension	1	95 °C	2 mins
3. cDNA synthesis	40	95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s	
4 (Dissociation)	1	95 °C for 10 s, 60 °C for 30 s, and 95 °C for 15 s*	

*+0.5°C/cycle, ramp 0.5 °C/s

3.3.15 RNA Preparation and Isolation

Using a Trizol™-chloroform extraction method (Simms et al., 1993), total RNA was isolated from whole bilateral adrenal tissue. Previously collected tissue was weighed into autoclaved 2 mL bead beating tubes (cat. #72.693.005, Sarstedt) that contained four 3 mm glass beads (cat. #11.312A, Thermo Fisher Scientific). Working on ice, exactly 1 mL of Trizol™ Reagent (cat. #15596026, Thermo Fisher Scientific) was added to bead beating tubes, and tissues were homogenized with one round of bead beating (6.0 m/s for 40 s, quick prep setting, FastPrep® 24 5G Homogenizer, MP Biomedicals). If any visible tissue particles remained, samples with beads were vortexed for 5 s, rather than repeating an aggressive bead beating round. After bead beating, tissue homogenates were transferred to Phase Lock Gel™ tubes (previously centrifuged for 30 s at 1,500 *g* at room temperature to settle gel to bottom; 2 mL, cat. #2302830, Quantabio) and 250 µL of chloroform was added. All tubes were shaken vigorously between two tube racks for 15 s before incubating at room temperature for 3 mins. Then, in order to separate out the Trizol™, tubes were centrifuged at 12,000 *g* for 10 mins (4 °C), leaving an upper aqueous phase containing uncleaned RNA.

Still working on ice, this aqueous phase was transferred to a new 1.7 mL microtube, and RNA was precipitated out by adding 500 µL of 2-propanol and mixing by repeated inversion. Samples were incubated at room temperature for 10 mins and centrifuged at 12,000 *g* for an additional 10 mins (4 °C) to pellet the RNA. Supernatants were carefully

aspirated, and pellets were washed by adding 1 mL of 0.22 μmicron filtered 70% ethanol (prepared with nuclease-free water, cat. #W4502-1L, Sigma-Aldrich). Tubes with added ethanol were then vortexed (so the pellet would dislodge) and re-centrifuged at 12,000 *g* for 10 mins (4 °C). The supernatant was carefully aspirated again, and pellets were left to dry (i.e., for ethanol to evaporate) in a biosafety cabinet for 1 hour. After pellets were dry, 50 μL of nuclease-free water (cat. #W4502-1L, Sigma-Aldrich) was added to tubes. Tubes were vortexed, quick-spun, and incubated at 56 °C (Fisherbrand™ Isotemp™ Digital Dry Bath, cat. #88-860-025) for 10 mins, with occasional inversion to dissolve the pellet in the water. Before RNA clean-up, RNA concentrations were spot-checked by NanoDrop (one sample per each of the eight groups; 1 μL RNA loaded; Thermo Scientific™ Nanodrop™ One Microvolume UV-Vis Spectrophotometer, cat. #ND-ONE-W). Concentrations ranged from 2391.7 ng/μL to 4561.7 ng/μL, with 260/280 ratios ranging between 1.94 and 1.99.

3.3.16 RNA Clean-Up and Quality Validation

For RNA clean-up, 350 μL of RLT buffer (part of the RNeasy Mini Kit 50, cat. #74104, QIAGEN) was first added to each tube and mixed by flicking. Then, 250 μL of 100% ethanol (0.22 μmicron filtered) was added and mixed by flicking. Mixed samples were transferred to spin column tubes (part of the RNeasy Mini Kit), centrifuged at 12,000 *g* for 1 min (room temperature), and outflow was discarded. Columns were placed back into the collection tubes, and 500 μL of RPE buffer (part of the RNeasy Mini Kit, prepared with 100% ethanol) was added. Then, tubes were centrifuged at 12,000 *g* for 1 min (room temperature), and outflow was discarded. Spin column tubes were placed into new collection tubes, and another 500 μL of RPE buffer was added. Tubes were centrifuged at 12,000 *g* for 2 mins (room temperature). Outflow was discarded, and columns were placed into new collection tubes and centrifuged at 12,000 *g* for 3 min (room temperature).

Next, columns were placed into new 1.7 mL microtubes, and 75 μL of RNase-free water was added. Tubes were incubated for 1 min (room temperature) and centrifuged at 12,000 *g* for 1 min (room temperature). Another 75 μL of RNase-free water was added to columns, and the incubation and centrifugation was repeated. Columns were discarded, and RNA concentrations were measured with the NanoDrop™ 8000 Spectrophotometer at a 1 in 5

dilution (with nuclease-free water, cat. #W4502-1L, Sigma-Aldrich) to ensure nanodrop readings were in range for reading (1 µL loaded; cat. #ND-8000-GL, Thermo Fisher Scientific). At this stage, RNA samples would ideally show a 260/280 ratio of 2.0 – 2.2 after the clean-up step (Thermo Fisher Scientific, 2009). It was found that RNA sample concentrations (i.e., at the 1 in 5 dilution) ranged from 103.0 ng/µL to 325.5 ng/µL (i.e., approximately 5 times lower than actual concentrations), with 260/280 ratios between 1.95 and 2.08. Next, the quality of the extracted RNA was examined; two RNA samples (also at the 1 in 5 dilution) per group (16 total) were randomly chosen to run on two bioanalyzer chips, as per the manufacturer's instructions (RNA 6000 Nano Kit, cat. #5067-1511, Agilent Technologies). The 16 RNA integrity numbers (RINs; for total eukaryote RNA) ranged from 5.00 to 8.70 (mean = 7.09; Supplementary Figures S3.1 and S3.2).

3.3.17 DNase Treatment and cDNA Conversion

To remove any genomic DNA contamination from the adrenal RNA samples, all samples were treated with a DNase cocktail comprised of 2 µL TURBO DNase 10X Buffer and 3 µL TURBO DNase (TURBO DNA-free™ kit, cat. #AM1907, Invitrogen, Thermo Fisher Scientific). Samples were normalized to 4 µg by adding specific volumes of cleaned RNA and nuclease-free water (cat. #W4502-1L, Sigma-Aldrich), calculated using the nanodrop concentrations (ng/µL) obtained (see Section 3.3.16) to achieve 4 µg in 30 µL total volume (including 5 µL of DNase cocktail). Normalization was performed with a liquid handling robot (ep*Motion*® *5075t* liquid handling robot, cat. #5075006022, Eppendorf). After normalization, sample tubes were vortexed and incubated at 37 °C for 25 mins. Then, 3 µL of DNase Inactivation Reagent (TURBO DNA-free™ kit, cat. #AM1907, Invitrogen, Thermo Fisher Scientific) was added. Samples were vortexed again, and a 5-minute room-temperature incubation was performed. A centrifugation at 12,000 *g* for 2 mins (room temperature) was performed, and exactly 21 µL of supernatant containing the RNA was transferred to a new microtube.

To verify that the DNase treatment worked, we performed qPCR of 16 DNase-treated RNA samples (2 samples per experimental group; 1 µL RNA used per sample, leaving 20 µL of

DNase-treated RNA remaining). A result of no amplification of RNA samples would indicate that genomic (g)DNA contamination was not present (i.e., that the DNase treatment worked). The CFX96 Touch™ Real-Time PCR Detection System (cat. # 1855196, Bio-Rad) was used, with primers (Table 3.4) that were reconstituted to 100 µM with nuclease-free water (cat. #W4502-1L, Sigma-Aldrich). To test the DNase-treated RNA samples, we used forward and reverse primers from reference gene RPL13a (Ribosomal Protein L13a), as optimization has shown that it has the earliest Cqs of the two reference genes used in this experiment (Table 3.4). Per reaction, master mix (24 µL per reaction) was comprised of 12.5 µL SYBR (5 mL SYBR™ Select Master Mix, cat. #4472908, Applied Biosystems, Thermo Fisher Scientific), 0.075 µL of forward primer (300 nM final concentration), 0.075 µL of reverse primer (300 nM final concentration), and 11.35 µL nuclease-free water (cat. #W4502-1L, Sigma-Aldrich).

After confirming that no gDNA was present, RNA was converted to cDNA with a reverse transcription protocol. First, RNA was normalized to 2 µg by performing a 1 in 2 dilution (10 µL RNA, 10 µL nuclease-free water) while working on ice. A reverse transcription cocktail master mix was prepared with 2 µL oligo dT (50 µM final concentration; 5' – TTTTTTTTTTTTTTTTTTTTTTTVN – 3') and 2 µL dNTP mix (10 mM final concentration; 100 mM dNTP set, cat. #10297018, Invitrogen, Thermo Fisher Scientific) per sample. Then, 4 µL of this reverse transcription cocktail was added to each microtube containing 2 µg of DNase-treated RNA. All samples were incubated at 65 °C for 5 mins, followed by a 1 min incubation on ice. Next, a reverse transcription enzyme cocktail was prepared as a master mix, with 4 µL 5X first strand buffer, 1 µL DTT (0.1 M), and 1 µL superscript IV (reverse transcription enzyme; all part of the SuperScript® IV kit, cat. #18090010, Invitrogen, Thermo Fisher Scientific). Per sample, 6 µL of this master mix was added, and a 50-minute incubation at 50 °C was performed. The reaction was terminated by a 5-minute 85 °C incubation. Finally, 1 µL of RNase mix (to remove any residual RNA in the cDNA samples) was added, and samples were incubated at 37 °C for 30 mins. The RNase mix was prepared as a master mix (400 µL total volume), with 1 µL PureLink™ RNase A (0.05 mg/mL final concentration; cat. #12091-021, Invitrogen, Thermo Fisher Scientific), 10 µL RNase H (0.05 U/µL final concentration; cat. #18021-014, Invitrogen, Thermo Fisher Scientific), and 389 µL nuclease-free water (cat. #W4502-

1L, Sigma-Aldrich). cDNA was stored at −20 °C until analysis.

On the previously mentioned DNase-treated RNA qPCR plate, we also took the opportunity to test all four of our primers (for reference and target genes; see Table 3.4) with pooled cDNA (pooled from eight samples, one rat per experimental group) at a 1 in 10, 1 in 100, and 1 in 1000 dilution (prepared with nuclease-free water). Testing the pooled cDNA had the goals of confirming we did have cDNA that would amplify with all primers and determining the best serial dilution range for standard curve creation for eventual RT-qPCR (i.e., to calculate PCR efficiency). NTCs for each master mix were added as negative controls, and rat gDNA (cat. #69238-3, Sigma-Aldrich) was tested at a 1 in 10, 1 in 100, 1 in 1000 dilution for each primer-specific master mix as both a positive and negative control. Specifically, this gDNA was isolated from rat plasma, so RPL13A and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) should amplify (positive controls), and NPY and GR primers should not amplify as these genes are not present in measurable amounts in plasma (additional negative controls). qPCR for the cDNA was conducted in a 25 µL reaction volume (22.5 µL master mix, 2.5 µL diluted cDNA). Per reaction, master mix was comprised of 12.5 µL SYBR (5 mL SYBR™ Select Master Mix, cat. #4472908, Applied Biosystems, Thermo Fisher Scientific), 0.075 µL of forward primer (300 nM final concentration), 0.075 µL of reverse primer (300 nM final concentration), and 9.85 µL nuclease-free water (cat. #W4502-1L, Sigma-Aldrich). Forward and reverse primers for both target (NPY and GR) and reference genes (RPL13A and GAPDH) are described in Table 3.4.

Table 3.4 Gene product sizes (base pairs) and sequences (forward and reverse) of primers for RT-qPCR target genes (i.e., neuropeptide Y and glucocorticoid receptor) and reference genes (i.e., glyceraldehyde-3-phosphate dehydrogenase and ribosomal protein L13a).

Gene Target (Accession #)	**Product Size (bp)**	**Forward Primer**	**Reverse Primer**
GAPDH (Z. Li et al., 2009; NM_017008)	184	5′ – CAG TGC CAG CCT CGT CTC ATA – 3’	5′ – TGC CGT GGG TAG AGT CAT A – 3’

Gene Target (Accession #)	Product Size (bp)	Forward Primer	Reverse Primer
RPL13A (Langnaese et al., 2008; NM_173340)	132	5′ – GGA TCC CTC CAC CCT ATG ACA – 3’	5′ – CTG GTA CTT CCA CCC GAC CTC – 3’
NPY (Shi et al., 2009; NM_012614)	288	5′ – GCT AGG TAA CAA ACG AAT GGG G – 3′	5′ – CAC ATG GAA GGG TCT TCA AGC – 3′
GR (Mashoodh et al., 2009; NM_012576)	188	5′ – GCT TCA GGA TGT CAT TAC GGG G – 3′	5′ – GCT TCA AGG TTC ATT CCA GCC – 3′

bp = base pairs; RPL13A = ribosomal protein L13a; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GR = glucocorticoid receptor; NPY = neuropeptide Y.

More specifically, RPL13A is a reference gene that encodes for the structural component of the 60S (large) ribosomal subunit, and GAPDH is a reference gene that encodes for a catalytic enzyme involved in glycolysis (Langnaese et al., 2008). RPL13A has been previously used by our group (Korgan et al., 2018) in hypothalamus samples from multiple ages of Long–Evans rats with a high-fat diet manipulation. The GR primer was designed and optimized by our group in a sample of Long–Evans rats (Mashoodh et al., 2009). We have also validated the use of RPL13A, GAPDH, and NPY with a small (n = 7) sample of rats of different ages given probiotic or no probiotic to verify that amplification of the reference genes is stable with probiotic treatment. In the optimization experiment, the NPY primer was noted to achieve amplification specificity, but the melt curve analysis was inconclusive with respect to how many products were being amplified (i.e., there was a clear peak but potential primer dimer formation). Thus, during the previous optimization, after RT-qPCR was performed, PCR was run on the cDNA, and this amplified product was analyzed on a gel. The gel confirmed that only one product was being amplified and was of the expected amplicon size of 288 bp (Supplementary Figure S3.3).

3.3.18 RT-qPCR for Neuropeptide Y and Glucocorticoid Receptor

Prior to RT-qPCR, cDNA was diluted 1 in 5 with nuclease-free water (cat. #W4502-1L, Sigma-Aldrich), using the ep*Motion*® *5075t* liquid handling robot (cat. #5075006022, Eppendorf). Subsequently, 384-well plates (clear shell/white well, cat. #HSP3805, Bio-Rad) were prepared with the liquid handling robot, with plates for each target (i.e., NPY or GR) balanced by experimental group. The first four plates contained 72 of 80 cDNA samples (i.e., 36 samples per plate, four or five samples per experimental group). Specifically, on each plate, all samples were added in triplicate for the specific target and both reference genes (i.e., 36 cDNA samples × three wells × three primers = 324 wells). The additional eight cDNA samples (one per each of the eight experimental groups) were added to four currently run 384-well RT-qPCR plates for another project (with 65 hippocampal cDNA samples, 36 or 37 samples total per plate) with the same target (NPY or GR) and reference genes (RPL13A and GAPDH). Additionally, on each of the eight plates, 10-fold serially diluted standard curves prepared from pooled cDNA (at 1 in 5, 1 in 50, 1 in 500, and 1 in 5000 dilutions, see Section 3.3.17) were added in duplicate for the three primer types per plate (i.e., GR/RPL13A/GAPDH or NPY/RPL13A/GAPDH), comprising an additional 24 wells per plate (i.e., eight wells with prepared master mix for each primer type). Finally, positive control cDNA and NTCs (master mix only) were added in duplicate for each primer type (i.e., four wells with prepared master mix for each primer type, 12 wells total), leaving the remaining wells as extra NTCs (i.e., 24 extra wells for the six plates with 36 cDNA samples, 15 extra wells for the two plates with 37 samples).

For all eight plates, RT-qPCR was conducted in a 10 µL reaction volume (9 µL master mix, 1 µL of 1 in 5 diluted cDNA template). Per reaction, specific primers (0.03 µL forward, 0.03 µL reverse; 300 nM final concentration each, see Table 3.4), along with SYBR (5 µL; 5 mL SYBR™ Select Master Mix, cat. #4472908, Applied Biosystems, Thermo Fisher Scientific), and nuclease-free water (3.94 µL; cat. #W4502-1L, Sigma-Aldrich) comprised each of the four master mix types (i.e., NPY, GR, RPL13A, and GAPDH). Cycling conditions are described in Table 3.3, with an annealing temperature of 60 °C for all primers. The CFX384 Touch™ Real-Time PCR Detection System was used to analyze plates (cat. #1855485, Bio-Rad). Amplification specificity was examined with

melt curves; melt temperatures were as follows: 81.50 for GR, 87.50 – 88.00 for NPY, 85.50 for RPL13A, and 84.50 for GAPDH. For each primer, PCR efficiencies were calculated on all eight plates for reference genes and on the four plates for each target (Supplementary Table S3.1). It should be noted that, for all primers, duplicate standard curves with four data points were attempted (i.e., pooled cDNA at a 1 in 5, 1 in 50, 1 in 500, and 1 in 5000 dilution). However, the fourth standard curve point for both targets (i.e., the 1 in 5000 dilution of pooled cDNA for NPY and GR) did not reliably amplify or amplified after the experimental cDNA, so all primer-specific PCR efficiencies are calculated from three points only (i.e., the 1 in 5, 1 in 50, and 1 in 500 pooled cDNA dilutions). This decision was made in order to calculate PCR efficiencies in the same way for all primers (i.e., targets and reference genes), so the 1 in 5000 standard curve duplicate was always removed. Because PCR efficiencies were calculated from the same three dilutions (duplicates) for all primers, Supplementary Table S3.1 shows that efficiencies for all four primers are similar (average of 113.44% for RPL13a, 112.83% for GAPDH, 112.80% for GR, and 113.33% for NPY), which is important for the acquisition of reliable RT-qPCR data (Bustin et al., 2009). Finally, triplicate experimental samples were checked for consistency using a Cq variation cut-off of 0.5 Cq (Ruiz-Villalba et al., 2021). If a triplicate varied by more than 0.5 Cq from either other replicate, that triplicate was removed, and the Cq mean was calculated from the remaining duplicates.

3.3.19 Hypothalamus Protein Extraction and Plasma Preparation

Prior to analysis of metabolic hormone levels in plasma**,** 10 µL of 10 mM DPP-IV (dipeptidyl peptidase-4) inhibitor (cat. #K4264-20MG, Sigma-Aldrich) was added to 100 µL of plasma. To prepare 5,000 µL of 10 mM DDP-IV inhibitor, 18.5 mg of DPP-IV inhibitor was added to 5,000 µL of 0.9% NaCl (sodium chloride; cat. #S8776-100ML, Sigma-Aldrich). DPP-IV inhibitor was added to increase levels of measurable bioactive GLP-1 because the DPP-IV enzyme degrades active GLP-1 (i.e., GLP-1[7 – 36]NH_2) to its primary metabolite (9-36NH_2) in plasma samples, and the Bio-Plex kit only measures the active form (i.e., it does not react with the amidated C-terminal [–COOH] in the way that assays that measure total GLP-1 do; Windeløv et al., 2017). Additionally, 10 µL of 1.3%

aprotinin (a protease, proteolytic enzyme, inhibitor) was added to each 100 µL plasma sample. To prepare this 1.3% aprotinin solution (cat. #A3428-100MG, Sigma-Aldrich), 100 mg of aprotinin was added to 7,500 µL of NaCl (cat. #S8776-100ML, Sigma-Aldrich).

For protein extraction from hypothalamus tissue, samples were weighed into autoclaved 2 mL bead beating tubes (cat. #72.693, Sarstedt) that contained four 3 mm beads (cat. #11-312A, Thermo Fisher Scientific). For 80 samples, cell lysis buffer cocktail was prepared with the following 50 mL recipe: 200 µL of cell lysis buffer factor 1 (cat. #9704161, Bio-Rad), 100 µL of cell lysis buffer factor 2 (cat. #9704162, Bio-Rad), 49.5 mL cell lysis buffer (cat. #9704159, Bio-Rad), and 200 µL of 500 mM phenylmethylsulfonyl fluoride (PMSF; cat. #P7626-250MG, Sigma-Aldrich) that was dissolved into Dimethyl Sulfide (DMS; cat. #D2650-100ML, Sigma-Aldrich). To prepare the PMSF in DMS (1,000 µL total volume), 0.0871 g of PMSF was added to 1,000 µL of DMS. For each sample, 1 mL of this cell lysis buffer cocktail was added to tubes and samples were bead beaded (MP FastPrep-24™ 5G homogenizer, MP Biomedicals) with the mouse brain protocol (i.e., one cycle at 6.0 m/s for 40 s, quick prep). After bead beating, supernatants were transferred into 1.7 mL microtubes to be centrifuged for 12,000 *g* for 10 min at 4 °C.

3.3.20 Bio-Plex Pro™ Rat Diabetes 5-Plex

All 80 plasma samples were analyzed with the Bio-Plex Pro™ Diabetes assay (i.e., leptin, active ghrelin, 29-amino acid pancreatic form of glucagon, plasminogen activator inhibitor-1 [PAI-1], and active glucagon-like peptide-1 [GLP-1]), as per manufacturer's instructions. Samples were diluted to 1 in 8 with Bio-Plex sample diluent. The standard curve was reconstituted with Bio-Plex standard diluent, as per manufacturer's instructions. Two plates were run that were counterbalanced for treatment, diet, and sex (four to five rats per each of the eight groups; 74 rats total) on both plates. As another lab member was running this assay at the same time, the remaining six samples that did not fit on the first two plates (one rat per six of eight experimental groups) were added to this third plate.

Data were analyzed with the Bio-Plex 200 system "high PMT, RP1" settings with DD Gates set at 5,000 (low) and 25,000 (high), with 50 bead events. Standard values were

included in analyses if the observed concentration divided by expected concentration was 1 ± 0.3 (70 – 130%). No standard duplicates had coefficients of variation (CVs) for their calculated concentration above 20%, but samples were excluded from further analysis for each of the specific analytes if they had CVs above 20%. As per an *a priori* decision, analytes were only included in analyses if they were measurable in 80% or greater samples (i.e., excluded if more than 20% of values were missing). In the case of analytes being 80% (or greater) measurable, if missing values were due to levels being below the standard curve, these missing values were replaced by the lowest standard curve calculated concentration on that plate. Overall, one sample was removed for leptin due to a CV above 20% and 11 samples for glucagon were replaced by the lowest standard curve value as they were not measurable on the standard curve.

3.3.21 Bio-Plex Pro™ Rat Cytokine 23-Plex

A total of 74 rats were split equally between two plates (nine to ten rats per each of the eight experimental groups) and counterbalanced for treatment, diet, and sex. The remaining six rats (one rat from six of eight experimental groups) were excluded because there was no current assay being run whereby these samples could be added (as was done in Section 3.3.20). The analytes measured by the 23-plex are G-CSF (CSF-3), GM-CSF (CSF-2), GRO/KC, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17A, IL-18, M-CSF, MCP-1, MIP-1α, MIP-3α, RANTES, TNF-α, and VEGF. Samples from each of the three plates were analyzed with the Bio-Plex Pro™ 23-plex assay as per manufacturer's instructions at a 1 in 4 dilution prepared with Bio-Plex sample diluent. For tissue lysate samples, Bio-Rad recommends adding bovine serum albumin (BSA) to 0.5% final weight by volume (w/v), so BSA stock solution that was prepared from molecular grade water (cat. #W4502-1L, Sigma-Aldrich) and lyophilized BSA powder (cat. #A9418-100G, Sigma-Aldrich) was added to each sample. Specifically, for a final volume per tube of 200 µL, hypothalamus lysates were prepared as follows: 10 µL of 10% BSA, 50 µL lysate, and 140 µL Bio-Plex sample diluent. On Bio-Rad's recommendation, the standard curve was prepared by adding the same 10% stock BSA to the sample diluent used to reconstitute (4,750 µL diluent, 250 µL 10% BSA to 0.5% final w/v BSA).

Data was analyzed with the Bio-Plex 200 system "high PMT, RP1" settings for Rat Cytokine (Group 1). Standard values were included in analyses if the observed concentration over expected concentration was 1 ± 0.3 (70 – 130%). No standard duplicates had CVs (concentration) above 20%, but samples were excluded from further analysis by analytes if they had CVs between duplicates above 20% (i.e., ten samples for GM-CSF, five samples for IFN-γ, one sample for IL-1α, one sample for IL-2, 13 samples for IL-7, two samples for IL-10, one sample for IL-12p70, and one sample for MCP-1). Again, a decision was made *a priori* to only include analytes with 80% or more analyte concentrations being measurable. As with the diabetes 5-plex, in cases where greater than 80% of samples were measurable, but some values were not measurable because levels fell below the bounds of the standard curve, these values were replaced by the lowest concentration on the standard curve. Analysis of each analyte's concentration in the hypothalamus sample lysates were normalized to total protein as measured by Bradford assay in duplicate (Bradford Reagent, cat. #B6916-500 mL, Sigma-Aldrich), with a standard curve of bovine serum albumin (cat. #A2153-10G, Sigma-Aldrich). Thus, data for each measurable analyte (i.e., 20 of 23 cytokines) are presented in fg of analyte per total protein in µg (fg/µg; Franklin & Perrot-Sinal, 2006; Myles et al., 2023).

3.3.22 Statistical Analyses

Statistical analyses were conducted with jamovi (Version 2.3.0; Fox & Weisberg, 2020; Lenth, 2020; R Core Team, 2021; The jamovi project, 2022), with GraphPad Prism (Version 9.2.0; GraphPad Software, San Diego, CA, USA) used to create graphs. Sample size (i.e., 80 rats, 10 per 8 groups) was determined from previous studies in our lab that have used diet and probiotic manipulations with similar outcome measures (Korgan et al., 2016, 2018; Mashoodh et al., 2009; Myles et al., 2023; Myles, O'Leary, Smith, et al., 2020). All dependent variables were analyzed by 2 (treatment; probiotic, placebo) × 2 (diet; Western, standard) × 2 (sex; male, female) factorial ANOVAs, or ANCOVAs where indicated. To further explore relationships between our dependent variables, Pearson's correlations were conducted between behavioural and physiological variables.

Alpha was set at .05 for ANOVAs, ANCOVAs, correlations, and *post hoc* testing (i.e.,

Tukey's test to probe significant interactions). Effect sizes are presented as η^2, with 0.01 or greater indicating a small effect, 0.06 or greater indicating a medium effect, and 0.14 or greater indicating a large effect (Lakens, 2013 from J. Cohen, 1988). Homogeneity of variance was analyzed with Levene's test; if Levene's test was significant (i.e., homogeneity of variance assumption violated), then a generalized linear model was conducted (IBM SPSS Statistics, version 28.0.1.1) to confirm ANOVA findings (summarized in Appendix C). All dependent variables were checked for outliers (see Table 3.5) and removed if greater or less than three standard deviations (*SD*) from the overall mean (*M*; Dunn, 2021). All outliers were removed for being greater than 3 *SD*s from the *M* (see Table 3.5), and no values less than 3 *SD*s from the *M* were found. Furthermore, Rat "N9" (a placebo WD female) was removed from all behavioural analyses due to not completing the entire 10-minute NSFT test, and rat "N1" (a placebo WD male) was removed from RT-qPCR analyses due to limited cDNA availability for amplification. All values were kept in RT-qPCR analyses as cDNA was normalized prior to analysis.

Table 3.5 Specific outliers that were removed, prior to conducting 2 × 2 × 2 factorial ANOVAs or ANCOVAs, for being greater than three standard deviations (*SDs*) from the overall mean (*M*).

Dependent Variable	Rat ID	Treatment	Diet	Sex
Adult Weight (g)	B1	Probiotic	Western	Male
Absolute/Relative kcal Intake	B1	Probiotic	Western	Male
	B2	Probiotic	Western	Male
Leptin (pg/mL)	B1	Probiotic	Western	Male
Ghrelin (pg/mL)	P6	Placebo	Standard	Female
Glucagon (pg/mL)	P1	Placebo	Western	Male
Unsupported Rears	Q9	Placebo	Standard	Female
Center Transitions	Q2	Placebo	Western	Male
Perimeter Pellet Contacts	T3	Placebo	Western	Male
Center Pellet Contacts	E10	Probiotic	Standard	Female
Calories Eaten in NSFT	J2	Probiotic	Western	Male
Home Cage Feeding Bouts	B1	Probiotic	Western	Male
Calories Eaten in Home Cage	Q1	Placebo	Western	Male
	T3	Placebo	Western	Male
Normalized IFN-γ Levels	E4	Probiotic	Western	Male
Normalized IL-2 Levels	E4	Probiotic	Western	Male
Normalized MIP-1α Levels	P1	Placebo	Western	Male

3.4 Results

3.4.1 Confirmation of Strain Presence in Moms and Offspring

The limit of detection for quantification of strain presence in caecum content samples was 1×10^4 CFU/gram of caecum contents for *L. rhamnosus* R0011 *and B. longum* R0175. The limit of detection for *L. helveticus* R0052 was 1×10^5 CFU/gram, as the 1×10^4 standard (duplicates) did not amplify. Even though the 10^4 standard did not amplify, all experimental probiotic rat samples (mothers and offspring) amplified for R0052 prior to the 10^5 standard. As depicted in Figure 3.5, R0011 (a concurrently used strain in the facility; part of the Lacidofil® probiotic) was not detected in any placebo, or probiotic, mothers (Figure 3.5A), or offspring (Figure 3.5B), in this experiment. Evidence of CEREBIOME® probiotic presence was detected in all probiotic animals tested and in no placebo animals. Furthermore, all extracted DNA from the caecum contents (placebo and probiotic; offspring and mother rats) showed amplification for the 16S rRNA gene (a positive control for the presence of bacterial DNA; Supplementary Figure S3.4).

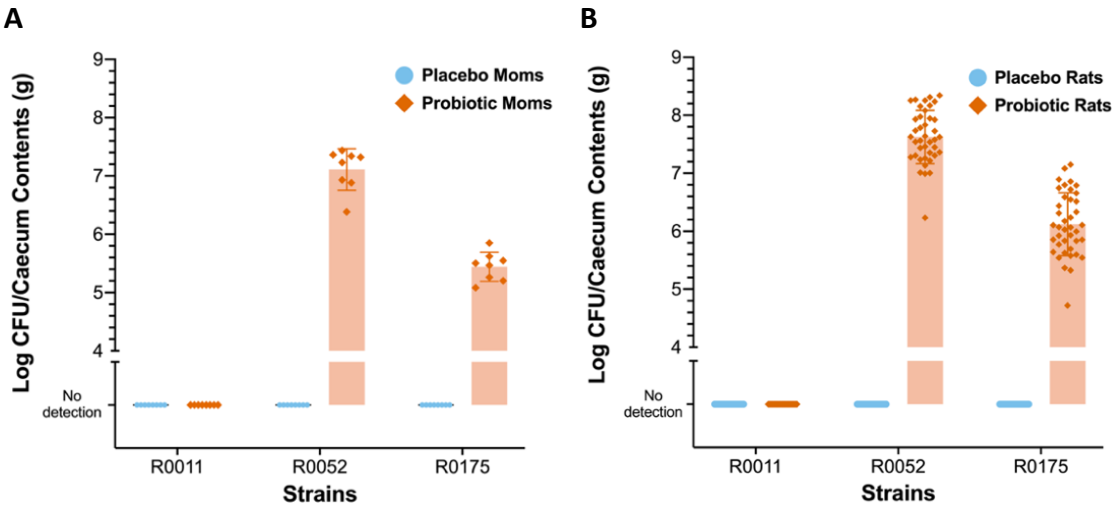


Figure 3.5 Graphical illustration of CEREBIOME® probiotic (i.e., *L. helveticus* R0052, *B. longum* R0175) presence (in log colony-forming units [CFU] per gram of caecum contents), along with *L. rhamnosus* R0011 presence in probiotic and placebo rats in the present experiment, broken down by **A.** Mothers ($n = 8$ probiotic, 8 placebo); and **B.** Offspring ($n = 40$ probiotic, 40 placebo).

Specifically, unspiked samples (see Section 3.3.13) were added to all plates as additional controls to give us an idea of where our experimental samples should be amplifying (i.e., ideally before the pooled unspiked control caecum samples). For mother rats, on the R0175 plate, the unspiked pooled caecum matrix from control animals amplified at a mean Cq of 31.51. No placebo mother rats had R0175 presence, and three of eight probiotic mother rats amplified below the unspiked control matrix, but five probiotic rat caecum samples amplified slightly above this threshold (mean Cqs from 31.67 to 32.92). This makes it difficult to confirm that R0175 was present in all rats at the time of sacrifice, but because we also measured R0052 presence and CEREBIOME® is comprised of 90% R0052 (10% R0175), we can confirm that the probiotic was administered on the day of sacrifice because all samples tested for R0052 presence amplified before the unspiked control matrix (mean Cq of 33.38).

For offspring, on the R0175 plate, the pooled unspiked control matrix amplified at a mean Cq of 31.48. Nearly all experimental samples amplified prior to this, but four rats had Cqs after this (mean Cqs of 34.24, 32.17, 31.89, and 31.49). For these four animals, we did also verify R0052 presence relative to the same unspiked control matrix. On the R0052 plate, the unspiked samples had a mean Cq of 33.61 and no offspring samples (including the previously mentioned four rats with low levels of R0175) amplified after the unspiked control matrix. For the R0011 plates (one for mothers, one for offspring), the unspiked control matrix amplified with a mean Cq of 34.35. As previously mentioned, no placebo or CEREBIOME® probiotic samples showed amplification for R0011.

3.4.2 Calorie Intake and Weight Measures

A treatment by diet by sex (2 × 2 × 2) factorial ANOVA on wean weight, at the start of diet and probiotic treatment, revealed that males (M = 48.20, SD = 5.82) weighed more at weaning (age P22) compared to females (M = 44.77, SD = 7.62; sex main effect, $F_{1,72}$ = 4.92, p = .030, η^2 = 0.062; Figure 3.6A). Rats did not differ by treatment or diet group; no other significant interactions were noted.

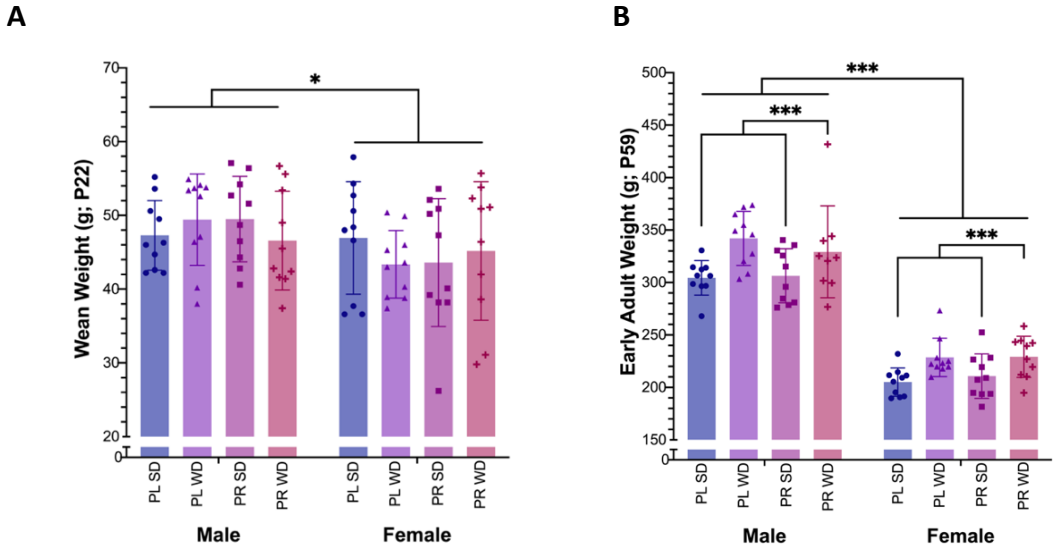
As wean weight was significantly affected by sex of the rats, early adult weight (P59) was analyzed by a treatment by diet by sex (2 × 2 × 2) factorial ANCOVA, covarying wean weight (wean weight was a significant covariate, $F_{1,71} = 51.63$, $p < .001$, $\eta^2 = 0.079$). The ANCOVA revealed a main effect of diet ($F_{1,70} = 43.48$, $p < .001$, $\eta^2 = 0.067$) and a main effect of sex ($F_{1,70} = 483.25$, $p < .001$, $\eta^2 = 0.741$). Specifically, WD animals ($M = 281.04$, $SD = 60.74$) had a higher early adult weight compared to SD animals ($M = 256.66$, $SD = 52.93$). As well, males ($M = 320.27$, $SD = 32.28$) had a higher early adult weight than females ($M = 218.42$, $SD = 20.73$; Figure 3.6B). There was no main effect of treatment on adult weight, nor any interactions between treatment, diet, or sex.

Another treatment by diet by sex (2 × 2 × 2) factorial ANOVA was conducted on daily calorie intake with intake per cage being divided by two (as rats were pair housed) to yield an absolute calorie intake value for each rat. The ANOVA revealed a main effect of diet ($F_{1,70} = 28.59$, $p < .001$, $\eta^2 = 0.063$), with WD rats ($M = 187.65$, $SD = 33.58$) consuming more calories than SD ($M = 172.34$, $SD = 30.14$) rats. The ANOVA further revealed a main effect of sex ($F_{1,70} = 337.93$, $p < .001$, $\eta^2 = 0.746$), with males ($M = 208.47$, $SD = 19.59$) consuming more calories than females ($M = 152.56$, $SD = 12.99$). There was a significant treatment by diet interaction revealed ($F_{1,70} = 9.68$, $p = .003$, $\eta^2 = 0.021$), but this interaction was superseded by a significant three-way treatment by diet by sex interaction ($F_{1,70} = 4.31$, $p = .042$, $\eta^2 = 0.010$; Figure 3.6C). In all groups, males consumed more calories than females (all $ps_{tukey} < .001$). Additionally, placebo WD males consumed more calories than both placebo SD males ($p_{tukey} < .001$) and probiotic WD males ($p_{tukey} = .018$). There was no difference by diet or treatment in females (all $ps_{tukey} > .05$), or between placebo and probiotic SD males ($p_{tukey} = .766$), or between SD and WD probiotic males ($p_{tukey} = 1.000$).

A (2 × 2 × 2) treatment by diet by sex factorial ANOVA on absolute daily calorie intake (kcal) by cage ($n = 39$ after one cage was removed as an outlier, a PR WD M cage) was also conducted. The ANOVA revealed a main effect of diet ($F_{1,31} = 12.66$, $p = .001$, $\eta^2 = 0.063$) and sex ($F_{1,31} = 149.66$, $p < .001$, $\eta^2 = 0.746$). Specifically, by cage, WD-fed rats consumed more calories ($M = 375.30$; $SD = 68.08$) than SD-fed rats ($M = 344.69$; $SD = 61.06$), and male rats ($M = 416.94$; $SD = 39.73$) consumed more calories than female rats ($M = 305.12$; $SD = 26.31$). The main effect of diet was superseded by a significant 2-way

treatment by diet interaction ($F_{1,31} = 4.29$, $p = .047$, $\eta^2 = 0.021$), whereby placebo SD rats (by cage $M = 338.63$; $SD = 56.66$) consumed fewer calories than placebo WD rats (by cage $M = 389.97$; $SD = 77.83$; $p = .002$). Of note, by cage, probiotic SD-fed rats did not differ in their calorie intake compared to probiotic WD-fed rats ($p_{tukey} = .730$).

As the calculation of absolute calorie intake assumes that rats are splitting food equally in the cage, individual calorie intake was also calculated relative to P59 weight. The average intake by cage and P59 weight were used to calculate a relative deviation of calorie intake from a 50:50 split (Myles et al., 2023). So, if cage mate rats were equal in weight, relative calorie intake would not differ from absolute calorie intake, but the more rats differ in weight, the more their calorie intake would deviate from 50:50. In the case of relative calorie intake, a treatment by diet by sex (2 × 2 × 2) factorial ANOVA again revealed a main effect of diet ($F_{1,70} = 21.49$, $p < .001$, $\eta^2 = 0.060$), with WD rats ($M = 187.65$, $SD = 34.39$) consuming more calories than SD rats ($M = 172.34$, $SD = 31.01$), and a main effect of sex ($F_{1,70} = 254.03$, $p < .001$, $\eta^2 = 0.710$), with males ($M = 208.47$, $SD = 20.91$) consuming more calories than females ($M = 152.56$, $SD = 14.97$). The main effect of diet was superseded by a significant treatment by diet interaction ($F_{1,70} = 7.28$, $p = .009$, $\eta^2 = 0.020$). *Post hoc* testing revealed that the only difference between treatment and diet groups was that placebo SD ate fewer calories than placebo WD rats ($p_{tukey} < .001$; Figure 3.6D).



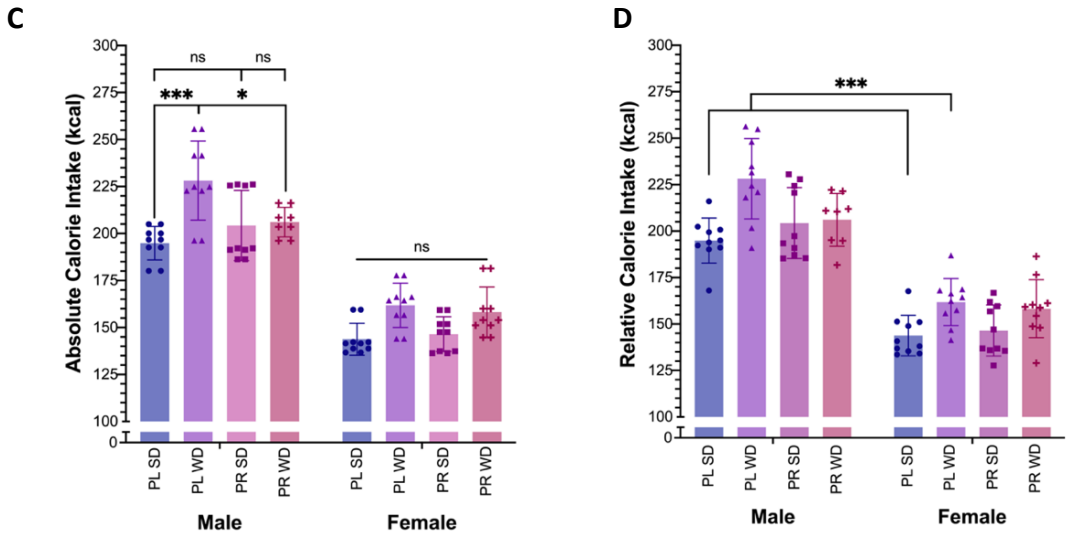


Figure 3.6 Graphical illustration of **A.** The main effect of sex on wean weight; **B.** The main effects of diet and sex on adult weight (covarying wean weight); **C.** The significant treatment by diet by sex interaction on absolute calorie intake; and **D.** The significant treatment by diet interaction on relative calorie intake. *$p < .05$, ***$p < .001$; ns = not significant, PL = placebo, PR = probiotic, SD = standard diet, WD = Western diet; data expressed as $M \pm SD$. *Note*: in Figures 3.6C and 3.6D, male rats always consumed more calories than females (i.e., in PL SD, PL WD, PR SD, and PR WD groups).

3.4.3 Anxiety-Related and Feeding Behaviours

Prior to testing rats in the NFST, 24-hour weight loss was recorded. A treatment by diet by sex (2 × 2 × 2) factorial ANOVA revealed a main effect of diet ($F_{1,72} = 102.05$, $p < .001$, $\eta^2 = 0.406$), whereby SD rats ($M = 27.78$; $SD = 8.48$) lost more weight with 24-hour food deprivation, compared to WD rats ($M = 16.18$; $SD = 5.34$), despite SD rats weighing less than WD rats. The ANOVA also revealed a main effect of sex ($F_{1,72} = 70.63$, $p < .001$, $\eta^2 = 0.281$), whereby males ($M = 26.80$; $SD = 7.98$) lost more weight than females ($M = 17.15$; $SD = 7.63$). With adult weight as a covariate (adult weight was a significant covariate, $F_{1,70} = 13.45$, $p < .001$, $\eta^2 = 0.061$), a treatment by diet by sex (2 × 2 × 2) factorial ANCOVA on fasting weight loss was conducted. The ANCOVA revealed that the main effect of sex was no longer significant ($F_{1,70} = 0.110$, $p = .742$, $\eta^2 = 0.001$), whereas the main effect of diet remained ($F_{1,70} = 128.76$, $p < .001$, $\eta^2 = 0.582$), and a diet by sex interaction appeared ($F_{1,70} = 6.34$, $p = .014$, $\eta^2 = 0.029$; Figure 3.7A). *Post hoc* testing of this interaction revealed

that SD males and SD females (p_{tukey} = .558) and WD males and WD females (p_{tukey} = .920) did not differ in weight loss. However, SD males lost more weight than WD males (p_{tukey} < .001), and SD females lost more weight than WD females (p_{tukey} < .001). Overall, *post hoc* testing of the interaction did not yield different conclusions from the main effect of diet, and so the meaningful conclusion is that SD rats lost more weight than WD rats.

Since the NSFT was conducted in a novel open field apparatus, a series of six treatment by diet by sex (2 × 2 × 2) factorial ANOVAs were conducted on anxiety-related behaviours in the OF apparatus. Since line crosses and supported rearing can depend on weight, additional treatment by diet by sex (2 × 2 × 2) factorial ANCOVAs were conducted with early adult weight as a covariate. In both cases, early adult weight was not a significant predictor in the ANCOVAs, indicating that, in this study, it does not significantly adjust the relationship between treatment, diet, and sex for line crosses ($F_{1,69}$ = 0.11, p = .738, η^2 = 0.001) or supported rearing ($F_{1,69}$ = 3.33, p = .072, η^2 = 0.038). Thus, six ANOVAs revealed main effects of treatment, diet and/or sex on line crosses, supported rears, unsupported rears, time in center, center entries, but no main effects or interactions on latency to enter the center (see Table 3.6).

Specifically, SD rats and males made fewer line crosses and supported rears compared to WD rats and females, respectively. Males also made fewer entries into the center than did females. Furthermore, placebo rats performed more unsupported rears and spent less time in the center of the apparatus compared to probiotic rats. Relatedly, WD rats spent less time in the center of the OF compared to SD rats. However, the main effects of treatment and diet on time in center were superseded by a significant treatment by diet by sex interaction ($F_{1,71}$ = 4.17, p = .045, η^2 = 0.044, Figure 3.7B). *Post hoc* testing revealed that the group driving this interaction was probiotic SD males, who spent more time in center than both placebo SD males (p_{tukey} = .012) and probiotic WD males (p_{tukey} = .029).

Table 3.6 Overview of treatment, diet, and sex main effects, in the 10-minute novelty-suppressed feeding task in a novel open field apparatus, for line crosses, supported rearing, unsupported rearing, center entries, and time in center (s), including means (*M*) and standard deviations (*SD*).

Behaviour	Main Effect	Mean (*SD*)		*F* value (df)	*p*-value	η^2
Line Crosses	Diet	*SD*	*WD*	4.08 (1,71)	.047	0.042
		133.60 (33.24)	148.72 (41.71)			
	Sex	*Males*	*Females*	21.28 (1,71)	< .001	0.217
		123.65 (37.70)	158.92 (29.79)			
Supported Rears	Diet	*SD*	*WD*	6.03 (1,71)	.016	0.061
		35.68 (10.26)	40.80 (10.45)			
	Sex	*Males*	*Females*	17.97 (1,71)	< .001	0.183
		33.78 (10.49)	42.74 (8.74)			
Unsupported Rears	Treat	*Placebo*	*Probiotic*	4.37 (1,70)	.040	0.056
		10.53 (5.44)	7.78 (5.84)			
Center Entries	Sex	*Males*	*Females*	7.33 (1,70)	.009	0.090
		17.64 (5.30)	20.97 (5.52)			
Time in Center (s)	Treat	*Placebo*	*Probiotic*	7.30 (1,71)	.009	0.077
		158.89 (70.00)	200.80 (78.30)			
	Diet	*SD*	*WD*	5.11 (1,71)	.027	0.054
		197.33 (90.47)	162.45 (55.42)			

Note. No main effects or interactions were revealed for the variable, latency to enter center; no interactions were revealed between treatment, diet, and sex for any anxiety-related variables. SD = standard diet; WD = Western diet.

In the NSFT, pellet contacts in the perimeter and center, pellet carries, latency to first eat the food in the center, feeding bouts, food weight eaten, and calories consumed during the 10-minute test were measured. A series of treatment by diet by sex (2 × 2 × 2) factorial ANOVAs were conducted, and main effects are summarized in Table 3.7. The ANOVAs revealed that probiotic rats made more center area contacts with the pellets and performed more feeding bouts than placebo rats. By diet, SD-fed rats had more feeding bouts than WD-fed rats, but WD-fed rats consumed more calories from food, with no difference in

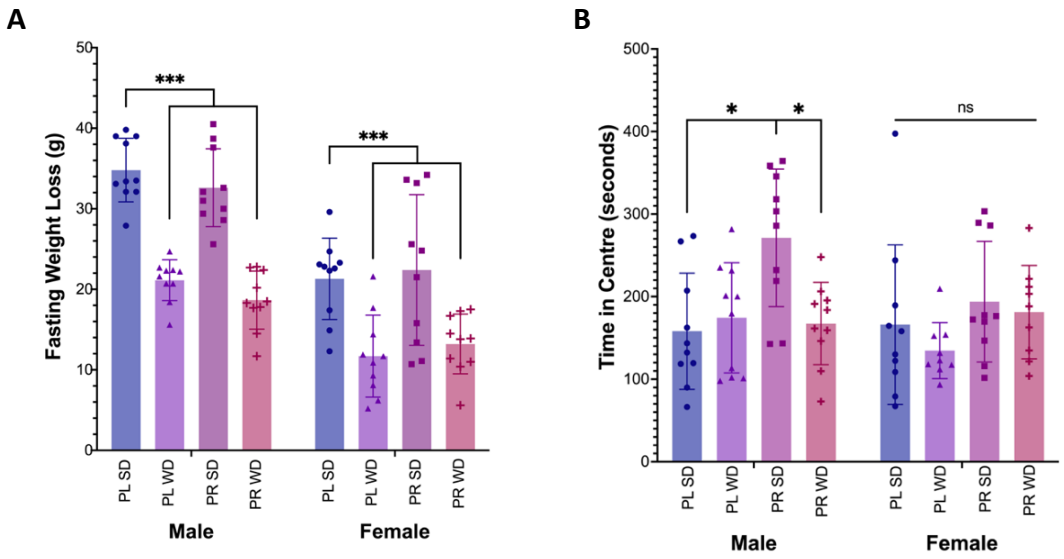
weight of food eaten. In the NSFT, there were also no group differences for perimeter pellet contacts, latency to first eat, or pellet carries.

Table 3.7 Overview of treatment, diet, and sex main effects on feeding-related behaviours during the 10-minute novelty-suppressed feeding task (NSFT) in a novel open field apparatus and 5-minute home cage (HC) feeding observation, including means (*M*) and standard deviations (*SD*).

Behaviour (Test)	**Main Effect**	**Mean (*SD*)**		***F* value (df)**	***p*-value**	**η^2**
Pellet Contacts (Center; NSFT)	Treat	*Placebo*	*Probiotic*	5.12 (1,70)	.027	0.063
		7.23 (5.22)	9.54 (3.64)			
Feeding Bouts (NSFT)	Treat	*Placebo*	*Probiotic*	4.81 (1,71)	.031	0.055
		3.44 (2.13)	4.50 (2.33)			
	Diet	*SD*	*WD*	7.34 (1,71)	.008	0.084
		4.63 (2.40)	3.31 (1.96)			
Calorie Intake (kcal; NSFT)	Diet	*SD*	*WD*	11.13 (1,70)	.001	0.124
		2.87 (2.12)	4.85 (3.22)			
Latency to First Eat (s; HC)	Diet	*SD*	*WD*	5.17 (1,72)	.026	0.055
		464.33 (80.49)	423.95 (88.57)			
	Sex	*Males*	*Females*	11.12 (1,72)	.001	0.119
		414.53 (82.16)	473.75 (81.30)			
Feeding Bouts (HC)	Sex	*Males*	*Females*	15.35 (1,71)	< .001	0.163
		3.69 (2.31)	1.98 (1.51)			
Food Eaten (g; HC)	Sex	*Males*	*Females*	22.25 (1,70)	< .001	0.219
		0.53 (0.39)	0.22 (0.18)			
Calories Intake (kcal; HC)	Sex	*Males*	*Females*	22.93 (1,70)	< .001	0.215
		2.18 (1.83)	0.83 (0.62)			

Note. No main effects or interactions were observed for amount of food eaten by weight (g), pellet contacts in the perimeter, latency to first eat, or pellet carries in the NSFT portion of testing. SD = standard diet; WD = Western diet.

In the 5-minute home cage observation that followed the NSFT, behaviours related to feeding (i.e., latency to first eat, number of feeding bouts, food weight eaten, calories consumed) were analyzed with a series of treatment by diet by sex (2 × 2 × 2) factorial ANOVAs. The ANOVAs revealed that SD animals took longer to first eat compared to WD animals, and females took longer to eat than males. As well, males had more feeding bouts and consumed more food (by weight and calories) compared to females (Table 3.7). However, these main effects of sex on food eaten and calories consumed were superseded by significant diet by sex interactions (food eaten, $F_{1,70} = 6.62$, $p = .012$, $\eta^2 = 0.065$; calorie intake, $F_{1,70} = 9.66$, $p = .003$, $\eta^2 = 0.090$). *Post hoc* testing showed that SD males and SD females, SD males and WD males, and SD females and WD females did not differ in food weight eaten, but WD males ate more food than WD females ($p_{tukey} < .001$; Figure 3.7C). As well, *post hoc* testing revealed that SD males and SD females, and SD females and WD females did not differ in calorie intake, but WD males had higher calorie intake than both WD females ($p_{tukey} < .001$) and SD males ($p_{tukey} = .010$; Figure 3.7D).



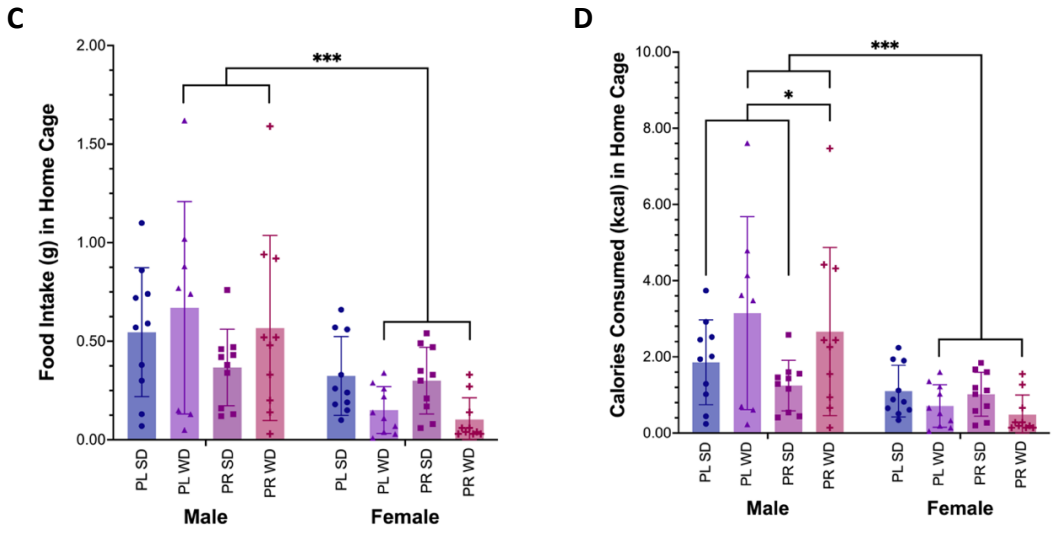


Figure 3.7 Graphical illustration of **A.** The significant diet by sex interaction (by ANVOCA, adult weight as a covariate) on 24-hour food deprivation-induced weight loss (g) before behavioural testing; **B.** The significant three-way interaction on time in center of the open field apparatus during the novelty-suppressed feeding task; **C.** The significant diet by sex interaction on food eaten in the home cage observation; and **D.** The significant diet by sex interaction on calories consumed during the home cage observation. *p < .05, ***p < .001; ns = not significant, PL = placebo, PR = probiotic, SD = standard diet, WD = Western diet; data expressed as $M \pm SD$.

3.4.4 Metabolic Hormone Levels in Plasma

In plasma samples, rat diabetes plex markers that were measurable in greater than 80% of samples were as follows: ghrelin, leptin, and glucagon. For leptin and ghrelin, all 80 plasma samples were measurable by the assay, although one sample for leptin was removed because the % CV was greater than 20% (a probiotic Western diet female). For glucagon, 13.75% of plasma samples read lower than the minimum point on the standard curve. Rather than include these values as zero, we changed the values to the lowest measurable standard curve point on their respective plates (i.e., 5 values were on plate 1; 6 values were on plate 2). GLP-1 was not measurable in 49 of 80 samples (with one measured sample having duplicates that varied by more than 20%; 50/80, 62.50% total missing). PAI-1 was not measurable in 63 of 80 samples (78.75% missing); thus, GLP-1 and PAI-1 analytes were not included in any further analyses. First, a 2 × 2 × 2 (treatment by diet by sex)

factorial ANOVA on levels of plasma glucagon did not reveal any main effects or interactions by treatment, diet, or sex.

Next, a 2 × 2 × 2 (treatment by diet by sex) factorial ANOVA on plasma levels of leptin revealed a main effect of diet ($F_{1,70} = 75.96$, $p < .001$, $\eta^2 = 0.450$) in that WD animals ($M = 5923.19$, $SD = 3140.73$) had higher plasma leptin compared to SD animals ($M = 1832.22$, $SD = 917.54$). Furthermore, there was a main effect of sex ($F_{1,70} = 10.44$, $p = .002$, $\eta^2 = 0.062$) in that males ($M = 4579.25$, $SD = 3756.87$) were higher in leptin compared to females ($M = 3071.26$, $SD = 1941.71$). These main effects were superseded by a diet by sex interaction ($F_{1,70} = 8.87$, $p = .004$, $\eta^2 = 0.053$), whereby SD males did not differ in leptin from SD females ($p_{tukey} = .998$), but WD males had higher leptin than both WD females ($p_{tukey} < .001$) and SD males ($p_{tukey} < .001$). As well, WD females had higher leptin than SD females ($p_{tukey} < .001$).

As weight and leptin levels are known to be highly positively correlated (Frederich et al., 1995), a 2 × 2 × 2 (treatment by diet by sex) factorial ANCOVA on plasma levels of leptin, with early adult weight as a covariate ($F_{1,69} = 4.014$, $p = .049$, $\eta^2 = 0.031$) was conducted. The ANCOVA still revealed a main effect of diet ($F_{1,69} = 47.05$, $p < .001$, $\eta^2 = 0.359$) and a diet by sex interaction ($F_{1,69} = 7.89$, $p = .007$, $\eta^2 = 0.060$; Figure 3.8A), but no longer a main effect of sex ($p = .642$). *Post hoc* testing revealed that, when factoring in early adult weight, WD males had higher leptin than SD males ($p_{tukey} < .001$), WD females had higher leptin than SD females ($p_{tukey} = .007$), but WD males were no longer significantly different in leptin from WD females ($p_{tukey} = .925$). In this case, *post hoc* testing of the interaction did not conclude anything different from the main effect, so the meaningful finding here is the significant main effect of diet (i.e., WD rats are higher in leptin compared to SD rats, even after controlling for body weight).

Another 2 × 2 × 2 (treatment by diet by sex) factorial ANOVA on plasma levels of ghrelin revealed a main effect of sex ($F_{1,71} = 11.99$, $p < .001$, $\eta^2 = 0.127$), whereby females ($M = 756.61$, $SD = 309.42$) had higher ghrelin than males ($M = 544.95$, $SD = 262.79$). This main effect of sex was superseded by a diet by sex interaction ($F_{1,71} = 6.85$, $p = .011$, $\eta^2 = 0.073$), whereby SD females had higher ghrelin than SD males ($p_{tukey} < .001$; Figure 3.8B). Unlike

with plasma leptin, a 2 × 2 × 2 (treatment by diet by sex) factorial ANCOVA on plasma levels of ghrelin with early adult weight as a covariate did not indicate that early adult weight was a significant covariate in the relationship between treatment, diet, and/or sex and plasma ghrelin ($F_{1,69} = 0.06$, $p = .807$, $\eta^2 = 0.001$).

3.4.5 Cytokine Levels in Hypothalamus

In extracted protein from hypothalamus tissue samples, inflammatory analytes measured by the 23-plex in greater than 80% of samples are as follows: G-CSF (CSF-3), GM-CSF (CSF-2), GRO/KC, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12(p70), IL-13, IL-17A, MCP-1, MIP-1α, MIP-3α, RANTES, TNF-α, and VEGF (i.e., 20 of 23). More specifically, IL-1β was not reliably measurable due to 19 sample duplicates having CVs above 20% and one value being above the bounds of the standard curve (27.03% missing values), and is, thus, excluded from further data analyses. Furthermore, M-CSF and IL-18 are excluded from analyses because greater than 20% of samples were not measured by the assay (i.e., concentrations were below the lowest point on their standard curves; 45.95% missing values for IL-18; 22.97% missing values for M-CSF).

Overall, a series of 2 × 2 × 2 (treatment by diet by sex) factorial ANOVAs consistently revealed that placebo animals showed higher normalized (to total protein) cytokine levels in hypothalamus tissue compared to probiotic animals (Table 3.8). Specifically, the analytes that were significantly higher in placebo animals compared to probiotic animals were G-CSF, GM-CSF, GRO-KC, IFN-γ, IL-5, IL-7, IL-10, IL-17A, MCP-1, MIP-3α, RANTES, TNF-α, and VEGF. No main effects of diet or sex, or interactions between treatment, diet, and sex were found, except for one treatment by diet interaction ($F_{1,60} = 5.91$, $p = .018$, $\eta^2 = 0.083$), whereby, irrespective of rat sex, placebo rats given SD had higher normalized hypothalamic IFN-γ than probiotic rats given SD ($p_{tukey} = .011$; Figure 3.8C).

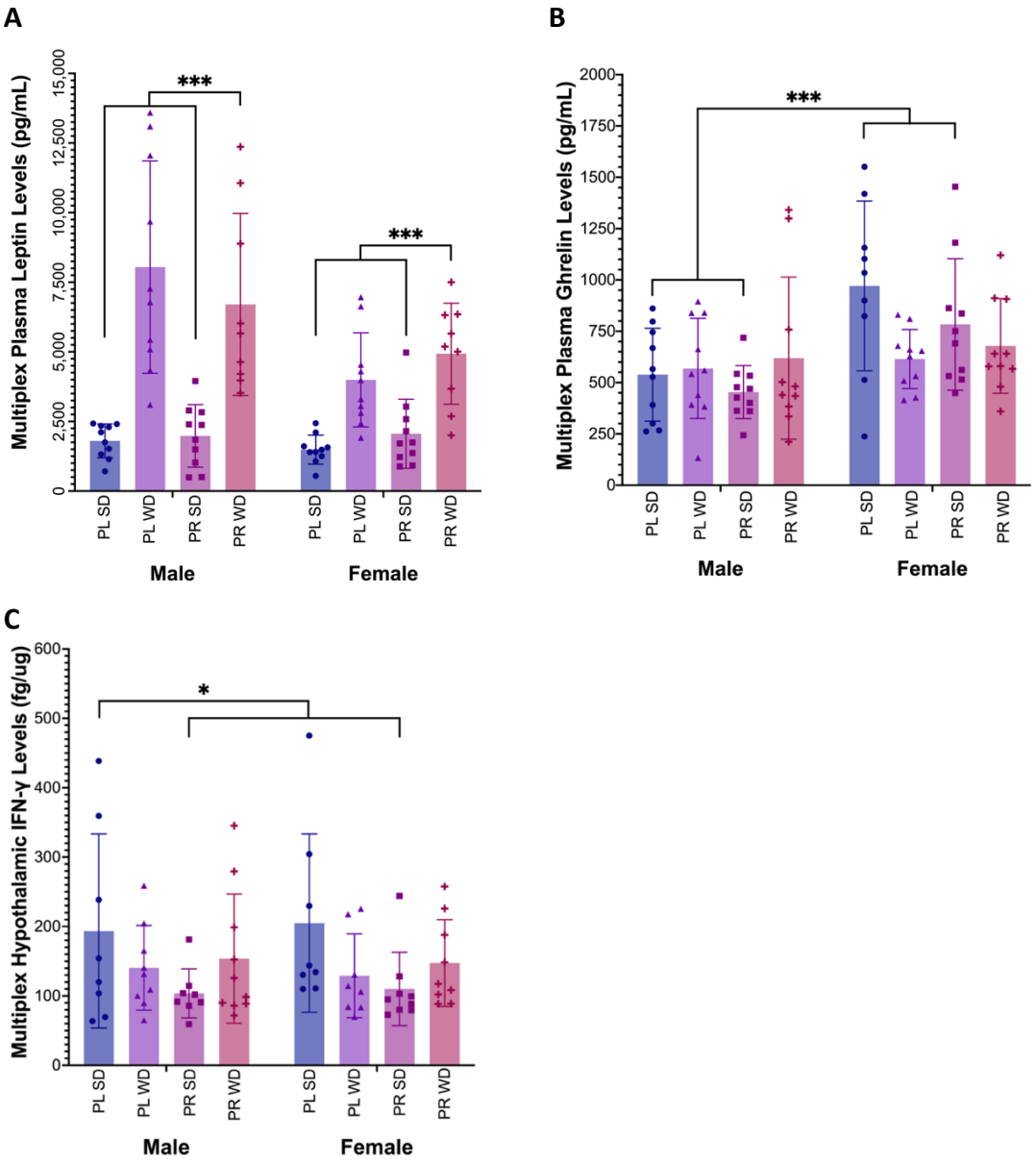


Figure 3.8 **A**. The significant diet by sex interaction (ANCOVA with adult weight as a covariate) on plasma leptin, as measured by the multiplex Luminex assay; **B.** The significant diet by sex interaction on plasma ghrelin levels; and **C.** The significant treatment by diet interaction on interferon-gamma (IFN-γ) levels (fg) in hypothalamus, normalized to total protein (µg). ***$p < .001$; ns = not significant, PL = placebo, PR = probiotic, SD = standard diet, WD = Western diet; data expressed as $M \pm SD$.

3.4.6 Neuropeptide Y and Glucocorticoid Receptor Expression

Of the 80 rat adrenal cDNA samples that were analyzed for NPY and GR expression, one sample (a placebo Western diet male) did not show amplification for either target or reference gene. Because nanodrop data indicated good-quality RNA after clean-up, it is likely that cDNA conversion did not work as intended for this sample, and this rat was excluded from RT-qPCR data analysis. Overall, gene expression data for NPY and GR target genes were normalized to two reference genes (i.e., GAPDH and RPL13A). Specifically, after averaging Cqs of triplicates for each sample (and removing any replicates that deviated from the other two by 0.5 Cq or greater; Ruiz-Villalba et al., 2021), the average of the Cqs for the reference genes (i.e., GAPDH and RPL13A) was subtracted from the Cq for the targets (NPY or GR) to yield ΔCq. For use in ANOVA, these ΔCq values (i.e., numbers on a logarithmic, log2, scale) were converted to their linear form ($2^{-\Delta Cq}$; Livak & Schmittgen, 2001).

A 2 × 2 × 2 (treatment by diet by sex) factorial ANOVA on NPY expression ($2^{-\Delta Cq}$) revealed a main effect of treatment ($F_{1,71} = 3.98$, $p = .0499$, $\eta^2 = 0.050$) in that the probiotic group ($M = 0.0445$, $SD = 0.0225$) was significantly higher in adrenal NPY expression than the placebo group (linear form of ΔCq, $M = 0.0351$, $SD = 0.0190$; The expression fold change (calculated with the $2^{-\Delta\Delta Cq}$ method; Livak & Schmittgen, 2001) for NPY (relative to the mean Cq of both reference genes) was found to be 1.27-fold higher in probiotic than the placebo adrenals (Figure 3.9C). There was no main effect of diet ($p = .899$) or sex ($p = .426$) on adrenal NPY gene expression, and no interactions between treatment, diet, and/or sex (all ps > .05).

Another 2 × 2 × 2 (treatment by diet by sex) factorial ANOVA on GR expression ($2^{-\Delta Cq}$) revealed a main effect of sex ($F_{1,71} = 8.40$, $p = .005$, $\eta^2 = 0.097$) in that females ($M = 0.0182$, $SD = 0.0045$) were significantly higher in adrenal GR expression than males ($M = 0.0154$, $SD = 0.0042$; Figure 3.9B). The expression fold change for GR was found to be 1.19-fold higher in females than males (Figure 3.9D). There was no main effect of treatment ($p = .819$) or diet ($p = .429$) on adrenal GR gene expression, and no interactions between treatment, diet, and/or sex (all ps > .05).

Table 3.8 Overview of treatment main effects for the inflammatory analytes measured in hypothalamic tissue sections (n = 74) by the Luminex 23-plex, normalized to femtograms (fg) of each analyte to micrograms (µg) of total protein in the sample, with significant p-values (i.e., < .05) in bold.

Analyte	Cytokine Category	Mean (*SD*)		*F* value (df)	*p*-value	η^2
		Placebo	Probiotic			
G-CSF	Regulatory (Panopoulos & Watowich, 2008)	1.49 (0.92)	1.15 (0.34)	4.50 (1,66)	**.038**	0.060
GM-CSF	CD131 Family/Regulatory (Hamilton, 2020)	43.45 (23.69)	32.02 (13.42)	6.17 (1,56)	**.016**	0.094
IFN-γ	IFN Family Cytokine (Akdis et al., 2011)	166.20 (103.47)	123.93 (56.93)	4.75 (1,60)	**.033**	0.066
IL-1α	IL-1 Family Cytokine (Akdis et al., 2011)	32.36 (22.23)	24.58 (7.46)	3.98 (1,65)	.050	0.055
IL-2	CD132/IL-2 Family Cytokine (Akdis et al., 2011)	296.87 (162.60)	234.78 (106.94)	3.51 (1,64)	.066	0.050
IL-4	CD132/IL-2/T_h2-like Cytokine (Akdis et al., 2011)	25.25 (16.84)	19.62 (5.86)	3.71 (1,66)	.058	0.051
IL-5	CD131/T_h2-like Cytokine Family (Akdis et al., 2011)	61.49 (37.49)	47.87 (13.16)	4.43 (1,66)	**.039**	0.059
IL-6	IL-6 Family Cytokine (Akdis et al., 2011)	86.99 (33.75)	75.24 (19.68)	3.41 (1,66)	.069	0.046
IL-7	CD132/IL-2 Family Cytokine (Akdis et al., 2011)	116.13 (64.46)	80.88 (37.84)	7.16 (1,53)	**.010**	0.115
IL-10	IL-10 Family Cytokine (Akdis et al., 2011)	127.34 (88.45)	91.23 (38.95)	5.58 (1,64)	**.021**	0.076
IL-12p70	IL-12 Family Cytokine (Akdis et al., 2011)	182.54 (118.91)	141.55 (55.45)	3.87 (1,65)	.053	0.053
IL-13	T_h2-like Cytokine Family (Akdis et al., 2011)	18.74 (13.73)	14.30 (4.79)	3.53 (1,66)	.065	0.048
IL-17A	IL-17 Family Cytokine (Akdis et al., 2011)	42.08 (30.71)	29.95 (10.04)	5.28 (1,66)	**.025**	0.070
MCP-1	CCR2 Receptor Chemokine (Griffith et al., 2014)	147.02 (101.30)	107.05 (48.06)	5.13 (1,61)	**.027**	0.072
GRO-KC	CXCR2 Receptor Chemokine (Griffith et al., 2014)	19.19 (12.14)	14.81 (4.42)	4.30 (1,66)	**.042**	0.058
MIP-1α	CCR1/5 Receptor Chemokine (Griffith et al., 2014)	2.66 (1.30)	2.20 (0.48)	3.94 (1,65)	.051	0.054
MIP-3α	CCR6 Receptor Chemokine (Griffith et al., 2014)	4.69 (3.02)	3.60 (1.16)	4.18 (1,66)	**.045**	0.057
RANTES	CCR1/3/5 Receptor Chemokine (Griffith et al., 2014)	35.34 (16.94)	27.87 (6.55)	6.37 (1,66)	**.014**	0.084
TNF-α	TNF Family Cytokine (Wallach, 2018)	244.25 (145.00)	192.23 (63.93)	4.10 (1,66)	**.047**	0.055
VEGF	VEGF Family Cytokine (Holmes & Zachary, 2005)	59.98 (45.01)	43.92 (15.86)	4.23 (1,66)	**.044**	0.057

G-CSF = granulocyte-colony stimulating factor (CSF-3); GM-CSF = granulocyte-macrophage colony-stimulating factor (CSF-2); GRO-KC = growth-regulated oncogene-keratinocyte chemoattractant (CXCL1); IFN-γ = interferon-gamma; IL = interleukin; MCP-1 = monocyte chemoattractant protein-1 (CCL2); MIP-3α = macrophage inflammatory protein-1 (CCL3); MIP-3α = macrophage inflammatory protein-3 (CCL20); RANTES = regulated upon activation, normal T cell expressed, and secreted (CCL5); TNF-α = tumour necrosis factor-alpha; VEGF = vascular endothelial growth factor.

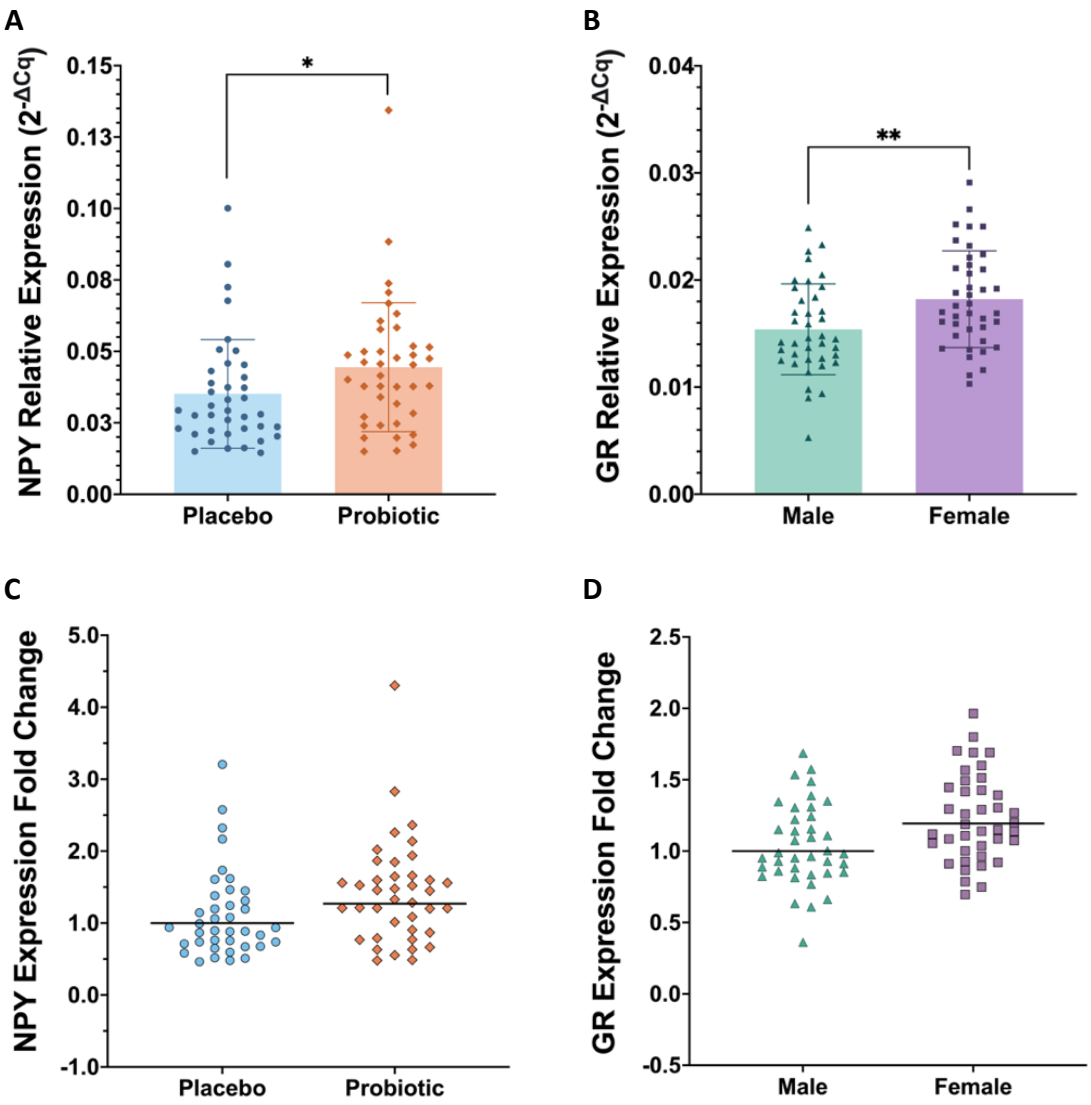


Figure 3.9 Graphical illustration of **A.** The main effect of treatment on the relative expression ($2^{-\Delta Cq}$) of neuropeptide Y (NPY) in placebo and probiotic rats; **B.** The main effect of sex on the relative expression ($2^{-\Delta Cq}$) of glucocorticoid receptor (GR) in males and females; **C.** NPY expression fold change ($2^{-\Delta\Delta Cq}$) of probiotic animals relative to placebo animals; and **D.** GR expression fold change ($2^{-\Delta\Delta Cq}$) of females relative to males. *p < .05, **p < .01; data expressed as $M \pm SD$ in 8A and 8B; horizontal bar is the geometric mean of the expression value, representing the fold change.

3.4.7 Relationships Between Metabolic and Behavioural Variables

Overall, early adult weight (age P59) was significantly correlated with relative calorie intake (i.e., P50 – 55 daily average, relative to P59 weight; $r = 0.978$, $p < .001$, $n = 78$).

This positive association was significant (*ps* < .001) in all subsets by independent variables (i.e., in placebo, probiotic, SD, WD, male, and female animals) and in each specific experimental group. While early adult weight was significantly positively correlated with 24-hour fasting weight loss overall ($r = 0.415$, $p < .001$, $n = 79$), and in placebo ($r = 0.439$, $p = .005$, $n = 40$), probiotic ($r = 0.390$, $p = .014$, $n = 39$), SD ($r = 0.793$, $p < .001$, $n = 40$), and WD ($r = 0.733$, $p < .001$, $n = 39$) animals, this correlation was not found to be significant when breaking groups down by sex. Furthermore, the only significant correlation between early adult weight and fasting weight loss (P63 – P69) by experimental group was with probiotic SD females ($r = 0.706$, $p = .023$, $n = 10$). Relative calorie intake and fasting weight loss were also significantly positively correlated overall ($r = 0.371$, $p < .001$, $n = 78$) and in probiotic ($r = 0.456$, $p = .004$, $n = 38$), SD ($r = 0.769$, $p < .001$, $n = 40$), and WD ($r = 0.671$, $p < .001$, $n = 38$) animals, but not in placebo animals, males, or females. By experimental group, relative calorie intake and fasting weight loss were significantly positively correlated only in probiotic SD males ($r = 0.766$, $p = .010$, $n = 10$) and probiotic SD females ($r = 0.633$, $p = .0499$, $n = 10$).

Correlations between sacrifice levels (P73 – P76) of metabolic hormones and adrenal NPY and GR gene expression with early adult weight and relative calorie intake are found in Tables 3.9 and 3.10. Broadly, early adult weight was negatively correlated with ghrelin, overall, and in placebo, probiotic, and SD subsets, but this relationship was not significant by experimental group (Table 3.9). As well, specific relationships between early adult weight and leptin levels or GR expression were apparent when examining these associations by experimental group. Specifically, early adult weight and leptin were positively correlated in placebo WD males and probiotic SD males, whereas adult weight and GR expression were negatively correlated in placebo WD females.

As detailed in Table 3.10, relative calorie intake was negatively correlated with ghrelin and GR expression but positively correlated with leptin. As with early adult weight, when examining these associations by specific experimental group, leptin and relative calorie intake were positively correlated only in placebo WD males and probiotic SD males. Further, in line with early adult weight results, relative calorie intake and GR expression were negatively correlated in placebo WD females. In contrast to early adult weight results,

there was a positive correlation between relative calorie intake and glucagon in probiotic WD males.

Table 3.9 Pearson's correlations between early adult weight and metabolic hormone (ghrelin, leptin, and glucagon) levels, GR gene expression, and NPY gene expression (n = 78), including all subsets by treatment, diet, and/or sex (n = 8 – 40).

Subset (*n*)	**Adult Weight & Ghrelin**	**Adult Weight & Leptin**	**Adult Weight & Glucagon**	**Adult Weight & GR Expression**	**Adult Weight & NPY Expression**
All rats (78)	-0.361**	0.444***	-0.150	-0.286*	-0.078
By treatment					
PL (39–40)	-0.337*	0.590***	0.113	-0.413**	-0.111
PR (38–39)	-0.398*	0.261	-0.377*	-0.109	-0.052
By diet					
SD (39–40)	-0.475**	0.274	-0.154	-0.135	0.053
WD (38–39)	-0.206	0.500**	-0.204	-0.470**	-0.240
By sex					
M (38–39)	0.071	0.502**	0.002	-0.017	-0.031
F (39–40)	-0.164	0.511***	-0.092	-0.065	0.086
By treatment, diet, and sex					
PL SD M (10)	0.442	0.507	0.389	-0.163	-0.171
PL SD F (9–10)	0.155	-0.170	-0.176	-0.148	-0.107
PL WD M (9–10)	0.288	0.798**	0.373	0.281	-0.008
PL WD F (10)	-0.029	0.054	-0.122	-0.655*	0.008
PR SD M (10)	-0.143	0.888***	0.067	0.306	0.458
PR SD F (10)	0.286	0.512	-0.448	-0.045	-0.137
PR WD M (9)	-0.213	-0.344	-0.665	-0.212	0.009
PR WD F (9–10)	-0.307	0.393	-0.150	0.044	0.443

*p < .05, **p < .01, ***p < .001; PL = placebo, PR = probiotic, SD = standard diet, WD = Western diet, M = male, F = female.

Table 3.10 Pearson's correlations between relative calorie intake and metabolic hormone (ghrelin, leptin, and glucagon) levels, GR gene expression, and NPY gene expression (n = 77), including all subsets by treatment, diet, and/or sex (n = 8 – 40).

Subset (*n*)	Relative Calorie Intake & Ghrelin	Relative Calorie Intake & Leptin	Relative Calorie Intake & Glucagon	Relative Calorie Intake & GR Expression	Relative Calorie Intake & NPY Expression
All rats (77)	-0.316**	0.526***	-0.127	-0.302**	-0.085
By treatment					
PL (39–40)	-0.217	0.705***	0.104	-0.396*	-0.095
PR (37–38)	-0.395*	0.266	-0.357*	-0.147	-0.056
By diet					
SD (39–40)	-0.462**	0.274	-0.193	-0.135	0.082
WD (37–38)	-0.103	0.648***	-0.154	-0.518**	-0.295
By sex					
M (37–38)	0.165	0.646***	0.081	-0.032	-0.053
F (39–40)	-0.138	0.544***	-0.151	-0.164	0.067
By treatment, diet, and sex					
PL SD M (10)	0.390	0.421	0.465	-0.076	-0.077
PL SD F (9–10)	0.139	-0.301	-0.301	-0.335	-0.004
PL WD M (9–10)	0.574	0.903***	0.218	0.188	-0.057
PL WD F (10)	-0.045	0.277	-0.254	-0.788**	0.040
PR SD M (10)	-0.326	0.805**	-0.118	0.147	0.542
PR SD F (10)	0.268	0.364	-0.458	-0.120	-0.181
PR WD M (8)	-0.237	-0.222	-0.761*	-0.601	-0.208
PR WD F (9–10)	-0.121	0.565	-0.171	-0.160	0.250

*$p < .05$, **$p < .01$, ***$p < .001$; PL = placebo, PR = probiotic, SD = standard diet, WD = Western diet, M = male, F = female.

Supplementary Table S3.2 overviews correlations between metabolic hormones and adrenal gene expression with fasting weight loss. Of specific note is that fasting weight loss and NPY expression were not correlated overall but were positively correlated in probiotic SD males ($r = 0.654$, $p = .040$, $n = 10$). As well, fasting weight loss was significantly negatively correlated with leptin, glucagon, and GR expression, but no significant correlations remained by experimental group.

Pearson's correlations were also conducted between early adult weight, relative calorie intake, fasting weight loss, and calories consumed in the NSFT and home cage (Supplementary Table S3.3). By experimental group, fasting weight loss was related to

calorie intake measured during behavioural testing, but only in three of eight groups. Specifically, fasting weight loss was negatively correlated with calories consumed in the NSFT in probiotic SD males (i.e., the more weight lost during the 24-hour fast, the fewer calories that were consumed during the NSFT; r = -0.670, p = .034, n = 10). Additionally, fasting weight loss was positively correlated with home cage calories in placebo SD males (r = 0.658, p = .038, n = 10) but negatively correlated with home cage calories in probiotic WD females (r = -0.705, p = .023, n = 10).

Additional correlations between metabolic and behavioural variables are provided as Supplementary Tables S3.4, S3.5, and S3.6. In Supplementary Table S3.4, Pearson's correlations between metabolic hormones (i.e., leptin, ghrelin, and glucagon), GR expression, and NPY expression were conducted in all rats, by both levels of each independent variable (i.e., placebo, probiotic, SD, WD, male, and female), and by each of the eight experimental groups. Of note, glucagon levels and GR gene expression were positively correlated only in placebo SD females (r = 0.754, p = .012, n = 10), whereas ghrelin levels and NPY gene expression were negatively correlated only in probiotic WD females (r = -0.715, p = .020, n = 10).

In Supplementary Table S3.5, Pearson's correlations are provided for the anxiety-related behaviours analyzed during the NSFT to overview differences in the relationships between measured anxiety-related behaviours by experimental group. For instance, line crosses and supported rears were significantly positively correlated (rs = 0.665 – 0.744, all ps < .05) in placebo SD males, placebo SD females, placebo WD females, and probiotic WD males, but not in the remaining four groups. In contrast, line crosses and center entries were significantly positively correlated (rs = 0.725 – 0.870, all ps < .05) in placebo WD males, placebo WD females, probiotic SD females, probiotic WD males, and probiotic WD females, but not in the remaining three groups.

Supplementary Table S3.6 provides a breakdown of Pearson's correlations for early adult weight, relative calorie intake, and fasting weight loss, with time to eat in the NSFT or home cage. No significant correlations were noted by the eight experimental groups, except that, as expected, calories consumed in the NSFT, or home cage, were often negatively

correlated with time taken to eat in the NSFT, or home cage, respectively. An overview of specific significant behavioural and metabolic correlations by experimental group is found in Figure 3.10. Of note, in placebo SD males, plasma ghrelin at sacrifice and calories consumed in the home cage observation were positively correlated, whereas ghrelin and calories consumed during the home cage observation were negatively correlated in placebo SD females. In placebo WD males, sacrifice levels of leptin were negatively correlated with calories consumed during the home cage observation, indicating that higher leptin at sacrifice was related to fewer calories consumed in the home cage in this group. In contrast, in probiotic SD males, adrenal NPY expression was negatively correlated with calories consumed in the NSFT, indicating that higher NPY at sacrifice was related to fewer calories consumed in an anxiety-inducing environment in this group. In probiotic WD males, adrenal NPY expression at sacrifice was positively correlated with calories consumed in the familiar home cage environment and the NSFT, suggesting that higher NPY is related to more calorie consumption regardless of the behavioural context.

3.5 Discussion

This work aimed to characterize behavioural and physiological outcomes of a probiotic formulation (i.e., CEREBIOME® or excipient placebo), in combination with a 'healthy' (i.e., SD) or 'unhealthy' (i.e., WD) dietary pattern, in both sexes of Long–Evans rats. With information from previous studies in this area, it was generally hypothesized that CEREBIOME® would foster behavioural and physiological health, whereas WD would impede behavioural and physiological health. Furthermore, we hypothesized that CEREBIOME® treatment and WD would interact in that CEREBIOME® would partially mitigate the negative health consequences of WD administration. Finally, males were expected to be more responsive to both the potentially adverse behavioural and physiological effects of the WD and the beneficial effects of the probiotic. To validate our probiotic manipulation, we confirmed presence of both the 16S rRNA gene (i.e., indicating bacterial DNA presence) in caecum samples from all mother and offspring rats in the experiment. Evidence of CEREBIOME® was detected in all probiotic-treated rats, and there was no cross-contamination of strains R0052 or R0175 in placebo-treated rats.

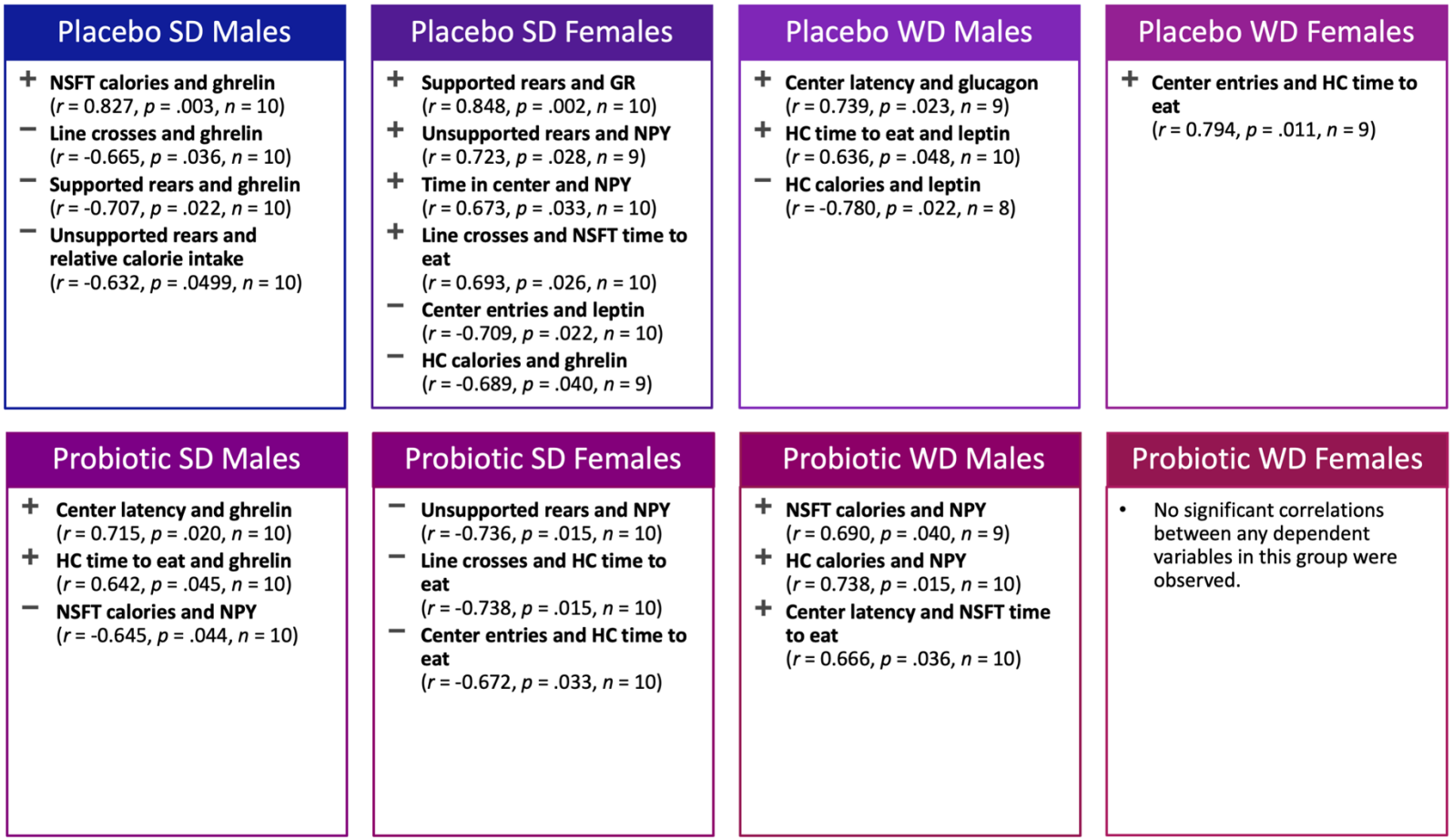


Figure 3.10 Summary of significant correlations between anxiety- and feeding-related behaviours, and metabolic measures (i.e., adult weight, relative calorie intake, leptin, ghrelin, NPY expression, and GR expression; n = 8 – 10). SD = standard diet, WD = Western diet, NSFT = novelty-suppressed feeding task, HC = home cage, GR = glucocorticoid receptor (gene), NPY = neuropeptide Y (gene).

Results for early adult body weight were analyzed by covarying wean weight as males weighed more at weaning than females, and wean weight was shown to be a significant covariate of early adult weight (as found previously in Myles, O'Leary, Smith, et al., 2020). As would be expected, in the present work, early adult weight was higher in animals fed a WD (compared to an SD) and in males (compared to females). Similar to the present results, we have previously shown that males and WD-fed rats weigh more than their respective counterparts, and females are not different in early adult weight by diet or treatment after an approximate period of seven weeks, starting at weaning (Myles, O'Leary, Smith, et al., 2020). However, another study with female C57BL/6 mice that administered the same WD as the present work for 16 weeks, compared to a purified HCD control, did report reduced body weight with additional probiotic treatment in the WD-fed rats (*Lactococcus* probiotic; 1×10^9 CFU/day; ATCC 19257; Naudin et al., 2020). In contrast to previous findings that probiotic treatment mitigates WD-induced weight gain in male rodents (Avolio et al., 2019; Myles, O'Leary, Smith, et al., 2020), we did not find that probiotic treatment influenced weight in early adulthood in the present work. Of note, Desbonnet and colleagues (2008) showed that in adult male Sprague–Dawley rats, treatment with *Bifidobacterium longum longum* 35624 via water bottles initially reduced daily body weight gain compared to placebo rats, but on day 14 of treatment, the probiotic-treated rats gained significantly more weight than the placebo rats. It could be that the time course of probiotic treatment and that different types of concurrently administered diets (e.g., different control diets) can alter findings with respect to body weight study-to-study.

With calorie intake, we report the same pattern of results as Avolio et al. (2019) and Myles, O'Leary, Smith, et al. (2020), whereby probiotic treatment normalizes calorie intake by diet in males (i.e., males fed the WD or HFD consume the most calories only when given the placebo or control). Specifically, relative to weight at the time calorie intake was measured, regardless of sex, placebo WD-fed rats consumed more calories than placebo SD-fed rats; further, probiotic-treated rats did not differ by diet in their calorie intake. In contrast to our previous findings reported in Myles, O'Leary, Smith, et al. (2020), that females given the probiotic consume more calories than females given the placebo, we did not find that females significantly differed in calorie intake by treatment or diet in this study after seven weeks of daily administration (i.e., ~50 doses of CEREBIOME®).

Similarly, about 48 doses (i.e., 16 weeks of treatment at three times a week) of a *Lactococcus* probiotic (1×10^9 CFU/day; ATCC 19257) to female mice did not affect the weight of food eaten of the same WD as the present work or its purified HCD control (Naudin et al., 2020). Overall, and correspondingly to Myles et al. (2023), we speculate that females require greater than six (as in Myles et al., 2023) or seven weeks (as in Myles, O'Leary, Smith, et al., 2020; the present work) of nutritional-related manipulations like probiotic or diet administration in order to better characterize differences that may emerge.

In the 10-minute NSFT in a novel open field apparatus, locomotion (i.e., line crosses, supported rears) and anxiety-related or exploratory behaviours (i.e., unsupported rears, centre entries, time in centre, and latency to enter center from the perimeter of the open field) were measured (see Table 3.1). By treatment, placebo rats performed more unsupported rears than probiotic rats, and probiotic SD-fed males spent more time in the centre than both placebo SD males and probiotic WD males. While we have previously reported that placebo-treated animals engage in more supported rearing than probiotic-treated animals during a 5-min open field test (OFT) and 5-min light-dark box (LDB) test (Myles, O'Leary, Smith, et al., 2020), we were not able to properly analyze unsupported rearing in that experiment because the frequency of the behaviour was low compared to supported rearing. It should be noted that a treatment difference in this present work appears for unsupported rearing, whereby placebo animals are performing more of this exploratory and potentially lower anxiety-related behaviour (Saxena et al., 2021; Sturman et al., 2018). With present results demonstrating that probiotic animals performed behaviours more indicative of a reduced anxiety-related state (e.g., time spent in center), this finding of placebo animals performing more unsupported rears is difficult to disentangle, especially because previous work suggests reduced anxiety-related behaviour with CEREBIOME® treatment. Specifically, administering the CEREBIOME® probiotic compared to placebo impacts locomotion and anxiety-related behaviours in both the OFT (e.g., fewer supported rears) and LDB (e.g., more transitions between light and dark; Myles, O'Leary, Smith, et al., 2020). As well, treatment with an 8-strain combination probiotic (administered in water bottles) to adult male Syrian golden hamsters resulted in fewer observed anxiety-like behaviours in the LDB (e.g., more time spent in light) and

elevated plus maze (EPM; e.g., more time spent in open arms), compared to animals who were not administered the probiotic (Avolio et al., 2019).

While no differences in latency to enter the center were revealed by treatment, diet, or sex, SD-fed rats and males made fewer line crosses and supported rears compared to WD-fed rats and females, respectively. Additionally, males made fewer entries into the center of the apparatus than females. It should be reiterated that adult weight was not found to be a significant covariate that affected line crosses or supported rearing in this group of animals. It is not unsurprising that females were more locomotory than males in this novel apparatus; this sex difference has been reported previously in Long–Evans rats in the EPM (i.e., open-arm entries; Ou et al., 2019), LDB (e.g., transitions between light and dark; Pirino et al., 2022), and OFT (e.g., line crosses and supported rearing; Myles, O'Leary, Smith, et al., 2020). At least in male rodents, chronic and acute stress have been shown to increase locomotor activity (e.g., more line crosses) and anxiety-related behaviours (e.g., less time in center in the OFT; Ieraci et al., 2016; Spasojevic et al., 2016). However, increased locomotion should not be interpreted as a reduced anxiety-related behaviour in the absence of other measures of reduced anxiety-related symptomatology, especially in female rodents. For instance, female mice have been reported to increase their locomotion (i.e., total distance travelled) when an OFT is conducted under stressful white light, whereas this difference is not observed in males (Sturman et al., 2018).

During the NSFT (i.e., the novel aversive situation), food intake (i.e., weight of food eaten, calorie intake, number of feeding bouts) and feeding-related behaviours (i.e., perimeter and center body contacts with the food pellet, pellet carries, latency to first feeding bout) were measured. In the NSFT, there was no difference by group for pellet contacts in the perimeter area, food intake by weight, time taken to first eat, or pellet carries. It should be highlighted that reduced latency to eat is the most common measure of lower anxiety in the NSFT (e.g., Bodnoff et al., 1988; Machado et al., 2013; Samuels & Hen, 2011), and we did not find a difference in this behaviour by treatment, diet, or sex. However, a reduction in the total amount of food eaten can also be used to infer an increased anxiety-related state (De Oliveira Sergio et al., 2021; Shephard & Broadhurst, 1982). We did observe that probiotic animals made more pellet contacts in the center of the open field and performed

more feeding bouts in any area than placebo animals. As well, SD-fed animals had more feeding bouts compared to WD-fed animals. In line with the general finding of reduced anxiety-related behaviours in probiotic- or SD-administered animals, more interaction with food in the NSFT is also indicative of lower anxiety-related symptomatology (De Oliveira Sergio et al., 2021).

During the 5-minute home cage observation (i.e., the familiar situation), we again measured food intake (i.e., weight of food eaten, calorie intake, number of feeding bouts) and feeding-related behaviours (i.e., latency to first feeding bout). Although we did not find any difference in latency to eat in the aversive NSFT apparatus by treatment, diet, or sex, we did find that males took less time to start eating in the familiar home cage and had more feeding bouts than females, which indicates a lower level of anxiety-related behaviour in the familiar environment (Samuels & Hen, 2011). Previous work has shown a sex difference with respect to feeding in the NSFT and home cage observation, with females being reported to interact less with the food, take more time to eat, and consume less food than males (De Oliveira Sergio et al., 2021). In contrast to the NSFT, where SD animals had more feeding bouts than WD animals, WD animals took less time to eat in the home cage, even though they had already consumed more calories than SD animals in the previous NSFT.

Because the NSFT protocol involves fasting rats for 24 hours prior to behavioural testing, we collected information on fasting weight loss in this study. Controlling for adult weight, both SD-fed males and females lost more weight over the 24-hour period than WD-fed males and females, respectively. In adult Long–Evans rats, a 24-hour fast is sufficient for significantly reducing body weight and body fat compared to pre-fast (Xian Liu et al., 2014), and in rodent studies, more body weight loss during a 24-hour fast can be a measure of increased metabolic rate (Forbes et al., 2001). In line with our findings, diet-induced (WD, compared to SD) obese adult male C57BL/6J mice have been previously reported to lose less body weight than SD-fed mice during a 24-hour fast (Ravinet Trillou et al., 2003). Additionally, both male and female mice without leptin receptors in NPY neurons (a model of obesity) have been reported to lose less body weight during a 24-hour fast than control mice (N. J. Lee et al., 2020). Interestingly, we did not observe a difference by sex in fasting

weight loss when controlling for body weight (ANCOVA has been reported to be preferred to using percentages in ANOVA; see Atkinson & Batterham, 2012); however, fasted adult female Long–Evans rats have been reported to lose less percent body weight compared to fasted males (Xian Liu et al., 2014).

When controlling for body weight, leptin levels in both sexes were found to be higher in WD-fed animals compared to SD-fed. Although we have previously reported, with a different type of control diet (i.e., a purified HCD), that probiotic-administered females were higher in plasma leptin than placebo-administered females (Myles, O'Leary, Smith, et al., 2020), we did not find a difference in leptin with probiotic treatment in either sex in the present work. In rodent models, increased leptin, alongside other markers of poor metabolic health, has been reported with HFD administration (Maniam & Morris, 2010). In such studies, increased leptin has also been linked to both reduced anxiety-like behaviours (maternally separated adult male Sprague–Dawley rats; Maniam & Morris, 2010) and increased anxiety-like behaviours (adult male Wistar rats given sucrose with chow; Rebolledo-Solleiro et al., 2017). Even though we did not find a sex difference for leptin levels after controlling for body weight, female rodents have been reported to show time-limited protection from increased weight and calorie intake after HFD exposure compared to males (Maric et al., 2022; Taraschenko et al., 2011). Furthermore, female mice do not become leptin resistant as quickly as males and will lose weight for longer if administered an HFD with leptin injections (R. B. S. Harris et al., 2003).

Paralleling leptin results, we did not find that CEREBIOME® treatment affected levels of plasma ghrelin at sacrifice. Research that studies leptin and ghrelin with probiotic treatment is somewhat limited, but in tilapia larvae, probiotic treatment with *Lacticaseibacillus rhamnosus* IMC 501® has been reported to affect the expression of leptin (i.e., reduce) and ghrelin (i.e., increase; Giorgia et al., 2018). Rodent studies suggest that ghrelin interacts with the stress response differentially based on sex, hunger status, and previous stress or diet exposure (e.g., see Carlini et al., 2002; Kristenssson et al., 2006; Saegusa et al., 2011); although human work has reported increases in ghrelin with stress exposure and anxiety disorder presence (McKay et al., 2021; Ozmen et al., 2019; Rossi et al., 2021). Previous work that measures plasma ghrelin levels with diet administration and no additional stress

manipulation in both sexes of rodent is limited. However, C. Yamada et al. (2020) reported a greater response to ghrelin administration in female C57BL/6J mice than in male mice (i.e., increased food intake without stress exposure, decreased food intake with novelty stress exposure in the females). Of note, another study reported that ghrelin-deficient male mice (C57BL/6J background) did not engage in increased WD intake (58% kcal fat, 26% kcal carbohydrate) in response to stress, compared to wild-type mice (Chuang et al., 2011). Further work on the effects of probiotic treatment and diet administration on leptin and ghrelin would benefit from including both sexes of rodent in experimental samples and administering diets and treatments for longer (e.g., at least 12 weeks) to ascertain if additional sex differences emerge.

In hypothalamus tissue protein, we found that placebo-administered animals had higher levels of various cytokines at sacrifice compared to probiotic animals (i.e., G-CSF, GM-CSF, GRO-KC, IFN-γ, IL-5, IL-7, IL-10, IL-17A, MCP-1, MIP-3α, RANTES, TNF-α, VEGF). Lower levels of inflammatory cytokines have been previously reported with the administration of bifidobacteria and lactobacilli probiotics to male rodents exposed to chronic stress (e.g., IL-6 in Mackos et al., 2013; IFN-γ in N. Li et al., 2018), but research that measures cytokine levels after probiotic treatment with no stress exposure at sacrifice is limited. Although we did not find a change in any cytokines with WD administration in the present work, we have previously reported that after predator odour stress, Long-Evans rats fed WD (D12079B) had increased plasma VEGF compared to control diet-fed (HCD; D14042701; Myles, O'Leary, Smith, et al., 2020). Further, there were specific interactions revealed between diet and sex (i.e., higher plasma IL-1β, IL-7, GM-CSF, GRO/KC, MIP-1α, and MCP-1 in Western-diet fed males) and diet and treatment (CEREBIOME® vs. placebo; i.e., higher plasma IL-7 and GM-CSF in placebo Western diet rats; Myles, O'Leary, Smith, et al., 2020). In a study with longer-term diet and probiotic administration without additional stress at sacrifice, Holowacz et al. (2015) reported that MCP-1 expression was increased in adipose tissue of 60% HFD-fed mice (adult male C57BL/6J mice, 14 weeks of diet, compared to SD) and decreased in HFD-probiotic-fed mice (5-strain combination *Bifidobacterium* and *Lactobacillus* strain, 1×10^9 CFU/day, five days a week for 14 weeks). However, these findings are difficult to disentangle because changes in cytokine levels may depend on length of diet or probiotic administration, the type of

control diet (e.g., SD or purified control), strain or sex of rodent, region of measurement (e.g., adipose tissue, hypothalamus, plasma), and sacrifice conditions (e.g., timing following stress exposure). Nonetheless, these limited studies on the interaction between probiotic treatment, diet, or stress exposure are reporting distinct alterations in inflammatory cytokine levels and further study into inflammatory alterations following nutritional interventions seems warranted.

Concerning IFN-γ, present findings report that placebo and probiotic rats were not different in levels of this analyte if given the WD, but placebo rats also fed the SD had increased hypothalamic IFN-γ compared to probiotic rats given the SD. IFN-γ function in the brain (e.g., communication with inhibitory neurons) has been reported to be reduced in rats that experience social isolation (Filiano et al., 2016). Interestingly, probiotic treatment (Lacidofil®, 6×10^9 CFU/day) to adolescent female C57BL/6 mice has been shown to reduce increased IFN-γ expression in the colon following *Citrobacter rodentium* infection (Gareau et al., 2011). Gareau et al. (2011) further reported that the probiotic treatment normalized memory impairment associated with the infection. Taken together, these somewhat limited studies suggest that IFN-γ has important behavioural, metabolic, and inflammatory functions in the brain and could be impacted by probiotic treatment, which warrants further investigation with additional experimental manipulations (e.g., stress exposure).

In the adrenal glands, we report significantly increased NPY expression in probiotic-treated animals compared to placebo animals. Apart from work by our laboratory group that found that probiotic animals were non significantly higher in adrenal NPY expression compared to placebo animals (Myles, 2019), there is no literature to date that has looked at NPY expression or protein changes in response to CEREBIOME® treatment**.** Previous work has been conducted demonstrating that *Lactobacillus* and *Bifidobacterium* probiotic strains can increase NPY protein and gene expression (e.g., *Lacticaseibacillus rhamnosus* GG and *B. lactic* Bb12 combined with the prebiotic inulin to adult male Sprague–Dawley rats in Lesniewska et al., 2006; *Lacticaseibacillus rhamnosus* IMC 501® to tilapia larvae in Giorgia et al., 2018). However, other work has reported no change in NPY expression (e.g., whole brains of adult zebrafish; Davis et al., 2016) and even reduced NPY expression (e.g.,

hypothalamus of male mice in Yadav et al., 2013; pancreas of male Sprague–Dawley rats with streptozotocin-induced type 2 diabetes mellitus [T2DM] in X. Zhou et al., 2021) with such probiotics. In contrast to some previous work, it should be highlighted that we did not find a difference in NPY expression by diet (e.g., reduced NPY expression in zebrafish fed an HFD; Falcinelli et al., 2017) or sex (e.g., female rats exposed to both brief maternal separation and cafeteria diet had reduced NPY compared to their SD-fed counterparts, with no difference in males; Maniam & Morris, 2010). Recent work also suggests that NPY expression in response to different experimental manipulations is sex- and tissue-specific. For instance, hypothalamic NPY expression is increased only in male *ob*/*ob* mice compared to control males, whereas adrenal NPY expression is decreased in both male and female *ob*/*ob* mice compared to same-sex controls (Werdermann et al., 2021). Overall, in the present work, we found increased adrenal NPY expression with CEREBIOME® treatment, but the relationship between NPY expression and nutritional manipulations warrants further investigation in different tissue types, animal models, and in both sexes.

With respect to GR expression in the adrenal glands, we found that females had increased expression compared to males. Previous research that studies sex-specific differences in adrenal GR expression is limited, but differences in the function of the adrenal glands in female mice compared to male mice have been described (i.e., complete tissue turnover in three months in female mice and nine months in male mice; Grabek et al., 2019). More specifically, this work determined that only female adrenal glands use specific stem cells for regeneration, and the authors mentioned that the physiological reasons for this sex-specific finding are unclear but that it would be relevant to study sex differences in adrenal hormone production (Grabek et al., 2019). While we did not find a difference in adrenal GR expression by treatment or diet in the present work, these factors have been shown to impact GR expression differentially by sex, at least in the brain. For instance, when exposed to both corticosterone (CORT) and lipopolysaccharide (LPS), regardless of the type of perinatal diet exposure (HFD or SD to dams for four weeks), female Long–Evans offspring had reduced GR expression in the amygdala, compared to females not exposed to CORT or LPS; however, in males, only the perinatally HFD-exposed had reduced GR expression in the amygdala, compared to males not exposed to CORT and LPS (Wijenayake et al., 2020). In general, male CD-1 mice have been reported to have increased

GR expression in the PVN compared to females (K. B. Smith et al., 2021). Furthermore, in the male mice only, treatment with a 5-strain lactic acid combination probiotic administered in kefir (3.0×10^9 CFU/day) has been reported to mitigate decreased GR expression in the PVN from treatment with LPS (K. B. Smith et al., 2021). Taken altogether, and in parallel to NPY findings, measuring the expression of metabolic- and stress-related genes remains limited, but it does appear that findings in the periphery cannot be generalized to the brain, and that results in males cannot be generalized to females.

As overviewed in detail in Section 3.4.7 (see Tables 3.9 and 3.10, Figure 3.10, and Supplementary Tables S3.2 – S3.6), many behavioural and molecular outcome measures were significantly associated, overall and in specific experimental groups. As would be expected, body weight and relative daily calorie intake were positively correlated overall and in each subset analyzed. As well, body weight and relative daily calorie intake were positively associated with plasma leptin levels, negatively correlated with plasma ghrelin and adrenal GR expression, and not found to be significantly correlated with adrenal NPY expression. Since this discussion cannot cover all interesting relationships, there will be a focus on linking back to the literature the significant correlations that were analyzed in each of the eight experimental groups. As will be discussed subsequently, these relationships between metabolic- or stress-related factors and anxiety-related behaviours are apparent in specific groups of rats that were administered the SD. In contrast, anxiety-related behaviours in WD-exposed rats were related to the presentation of feeding-related behaviours (e.g., time to first eat, calorie consumption; see also Figure 3.10). By sex, plasma leptin and adrenal NPY expression were related to metabolic parameters like body weight or calorie intake in various groups of males, whereas it was plasma ghrelin and adrenal GR expression in WD-fed females (i.e., probiotic- or placebo-administered) that were related to metabolic parameters.

Although leptin and body weight, along with leptin and relative daily calorie intake, were correlated overall, these relationships were only significant in placebo WD males and probiotic SD males. There was also a relationship between greater fasting weight loss during behavioural testing and higher adrenal NPY expression at sacrifice, but only in probiotic SD males. This relationship was not significant when examining all rats. In both

male and female mice, fasting has been reported to increase NPY expression in the hypothalamus and adrenal glands (Chua et al., 1991), but in the present work, NPY expression was analyzed approximately one-week post-fast, so this experimental difference means that conclusions are limited. It is interesting that plasma leptin was not found to be correlated with adrenal NPY expression in any groups in the present work because previous studies with HFD exposure to Sprague-Dawley rats have reported that lower plasma leptin is related to increased hypothalamic (Maniam & Morris, 2010) and increased hypothalamic NPY protein levels (Hansen et al., 2003; Velkoska et al., 2005).

It was found that lower adrenal GR expression was related to higher body weight and more daily calorie intake when examining all rats, but this relationship was only significant in placebo WD females. Likewise, higher plasma ghrelin was associated with higher adrenal NPY expression overall, but this relationship was only significant in probiotic WD females. In *db*/*db* male mice, increased GR expression in the liver has been associated with symptoms of T2DM, including increased CORT, insulin, and glucose in the blood (Y. Liu et al., 2005). Adolescent C57BL/6J mice exposed to acute stress have also been reported to have reduced food intake, hypothalamic NPY expression, and plasma ghrelin (Saegusa et al., 2011). However, it should be noted that with different types of stress exposure, different rodent strains, and in females, ghrelin has been reported to be increased with anxiety presence (e.g., Kristenssson et al., 2006). Overall, both ghrelin and NPY are orexigenic hormones, but where NPY is well-established as being anxiolytic (e.g., Cohen et al., 2012; Sajdyk et al., 2004), the role of ghrelin in mediating the stress response and anxiety-related behaviours in females compared to males is still somewhat uncharacterized.

In specific groups of females, many behavioural variables were correlated with hormone levels and adrenal gene expression (see Figure 3.10). Interestingly, leptin levels were not found to be correlated with most of the anxiety-related behaviours (see Table 3.1), but in placebo SD females, center entries and leptin were found to be negatively associated, indicating that more center entries are related to lower levels of leptin in this group. Leptin has been reported to be anxiolytic when administered (e.g., adult male C57BL/6J mice in the EPM; J. Liu et al., 2010), but in obese male and female rats, higher leptin has been associated with greater anxiety-related behaviours (Alonso-Caraballo et al., 2019).

Although the females in the present work who were fed the SD and placebo did not have any indicators of obesity (e.g., no increased leptin, weight, calorie intake), present interpretations are complicated by the motivational factor of having food in the center of the testing apparatus following a fasting period. Additionally, placebo SD females were not found to show reduced anxiety compared to the other groups, so it is difficult to draw conclusions about the negative association between center entries and leptin levels.

Different behavioural and endocrine outcomes were further related in placebo SD females (i.e., positive associations between supported rears and adrenal GR expression, unsupported rears and adrenal NPY expression, and time in center and adrenal NPY expression). In contrast, in probiotic SD females, more unsupported rearing was associated with lower NPY expression. CRF (an anorexigenic/anxiogenic peptide) is stimulated by neuropeptide-Y (an orexigenic/anxiolytic peptide), and both peptides are critical for the regulation of stress, anxiety, and feeding (Charmandari et al., 2005; Sajdyk et al., 2004; Schwartz et al., 1996; Thorsell, 2010). GRs directly encourage the negative feedback of the HPA axis (e.g., reduce CRF release from the PVN; de Kloet et al., 1998), and increased GRs in rats, at least in the hippocampus, has been associated with reduced anxiety-related behaviours (Sampedro-Piquero et al., 2014). Likewise, intracerebroventricular NPY injection increases time spent in the open arm of the EPM (male Sprague–Dawley rats; Heilig et al., 1989) and reduces latency to eat in the NSFT (adult female C57BL/6 mice; Antunes et al., 2015). Furthermore, Karl et al. (2008) report that NPY knockout mice of both sexes showed reduced exploration behaviour (hole-board test) and locomotion (various tests), along with increased anxiety in the EPM, OFT, and LDB. Overall, it is interesting that both higher adrenal GR and NPY were associated with more locomotory and reduced anxiety-related behaviours in groups of females, as the NSFT with food deprivation is an acute stressor that could impact both stress and feeding hormones.

3.6 Conclusions

This work provides research support for the idea that the administration of the CEREBIOME® probiotic improves anxiety-related outcomes and reduces cytokine levels in the hypothalamus of Long–Evans rats, as has been shown previously in rodent studies

(e.g., Messaoudi, Lalonde, et al., 2011; Mohammadi et al., 2019; Myles, O'Leary, Smith, et al., 2020; Partrick et al., 2021). In line with previous work (e.g., Avolio et al., 2019; Myles et al., 2020), CEREBIOME® treatment also mitigated the increased calorie intake from WD exposure. Interestingly, although in WD-fed animals, CEREBIOME® reduced calorie intake, the expression of the orexigenic factor, NPY, in the adrenal glands was found to be increased in probiotic-treated animals. This finding warrants further investigation (e.g., concurrent measurement in the hypothalamus and adrenals) because of the anxiolytic function of NPY and the fact that CEREBIOME® has been reported to reduce anxiety-related symptomatology, even in humans (Diop et al., 2008; Messaoudi, Lalonde, et al., 2011). As would be expected from previous work (e.g., J. A. S. Gomes et al., 2020; S. Lin et al., 2000; Maffei et al., 1995; Myles et al., 2023), increased leptin in response to WD administration was found in the present work, but importantly, this relationship was observed in both sexes. In contrast, a sex difference for plasma ghrelin was found, whereby females fed the SD had higher ghrelin than their male counterparts, but there was no sex difference with WD administration. These sex differences are important to characterize when studying behavioural and physiological markers of health, especially considering that females had increased adrenal GR expression. At least in the brain, increased GR expression could be protective with respect to anxiety- and stress-related outcomes and metabolic parameters (Ait-Belgnaoui et al., 2018; Crumeyrolle-Arias et al., 2014), although it should be stressed that research on adrenal GR expression changes in either sex is extremely limited.

Some limitations of this work should be highlighted, including not having more metabolic-related measures (e.g., insulin, glucose tolerance, adipose tissue weight or distribution), measuring anxiety-related behaviours with only one behavioural test, not measuring gene expression of NPY and GR in both the brain and periphery, and not measuring other markers of HPA axis function (e.g., CORT, ACTH) or sex hormones (e.g., estradiol). It has been recently reported that probiotic treatment with a *Lactococcus* probiotic improved glucose tolerance (i.e., time taken for blood glucose to normalize after glucose challenge) and mitigated weight gain and total serum cholesterol increases associated with the WD (Naudin et al., 2020). Interestingly, in this study, treatment with another probiotic (*Lacticaseibacillus rhamnosus* GG) did not significantly reduce body weight, total

cholesterol, or glucose tolerance in WD-fed rats (Naudin et al., 2020), highlighting the importance of considering that different probiotics have different effects on experimental subjects. While no differences in glucagon levels were found in the present work, measuring glucagon after a fast, along with insulin and blood glucose, in future work could yield noteworthy findings. In fact, adult male Wistar rats with streptozotocin-induced T2DM had reduced plasma glucagon and blood glucose after a two-hour fast when given a *Lactobacillus johnsonii* probiotic for two weeks in drinking water (Yamano et al., 2006).

The present work is complimented by previous studies that administered CEREBIOME® to male C57BL/6J mice and found reduced plasma CORT alongside reduced anxiety-related behaviours (Ait-Belgnaoui et al., 2014, 2018), but further studies that examine specific markers of HPA axis function in different strains of rodents and in females is warranted. Despite the need for future work with additional behavioural and physiological measurements that includes both sexes of rodent and that administers probiotic and diet for longer, our study is vital for research in this area because it provides information on the health-related effects of CEREBIOME® in females. Findings demonstrate behavioural and physiological alterations with probiotic treatment and WD administration, differentially in each sex of Long–Evans rat. These results have implications for increasing understanding of the development of and potential treatment options for human obesity and anxiety symptomatology.

3.7 Declaration of Competing Interest

EMM (IT11380, IT15513) and MEO (IT11379, IT15514) have held a total of four Mitacs Accelerate Fellowships with TSP via a collaboration with the Rosell® Institute for Microbiome and Probiotics (RIMaP; Lallemand Health Solutions Inc.). Specifically, IT15513 (PhD level to EMM) and IT15514 (PhD level to MEO) were active during this work. Both of these Mitacs grants allowed IDR and SIH to be employed in a research assistant capacity for the duration of the project. Mitacs Accelerate fellowships are a partnership that relies on both financial and in-kind contributions to graduate student research projects from industry. AP is an employee of the RIMaP (with SB) and aided in

protocol optimization and molecular work after the animal experiment portion of the project was complete. The CEREBIOME® probiotic and placebo formulations were kindly provided by Lallemand Health Solutions Inc. from the RIMaP. However, the RIMaP and Lallemand Health Solutions Inc. were not involved in any data analyses or manuscript preparation. During the completion of this project, additional financial support was awarded to EMM and MEO as PhD stipends from the Department of Psychology & Neuroscience and the Faculty of Graduate Studies at Dalhousie University, PhD level Nova Scotia Graduate Scholarships, and PhD level National Sciences and Engineering Research Council (NSERC) of Canada Awards (CGS-D to EMM, PGS-D to MEO). No other conflicts of interest are reported.

3.8 Funding

This project was directly supported by the one PhD level Mitacs Accelerate grant (IT15513) awarded to TSP and EMM and in-kind contributions from the RIMaP during molecular analyses. These in-kind contributions included the Luminex assay kits, reagents for extractions (DNA, RNA, and protein), qPCR protocols and optimization for strain detection, and Eppendorf liquid handling robot consumables and other RT-qPCR-associated reagents for NPY and GR gene expression analysis.

3.9 Acknowledgements

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3.10 CRediT Authorship Contribution Statement

EMM: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, validation, visualization, writing – original draft. **MEO**: conceptualization, data curation, investigation, methodology, project administration, validation, writing – review & editing. **IDR:** data curation, investigation, writing – review & editing. **SIH**: data curation, investigation, writing – review & editing. **LD:** formal analysis, investigation **AP:** formal analysis, investigation, project administration, resources, software, validation. **SB:** funding acquisition, project administration, resources. **TSP:** conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, supervision, writing – original draft/review & editing.

CHAPTER 4 DISCUSSION

4.1 Overview of Findings

In a male and female Long–Evans rat model, the research presented in this dissertation highlights distinct health-related behavioural and molecular alterations from probiotic treatment (i.e., CEREBIOME®, Lallemand Health Solutions Inc.) and WD administration (i.e., D12079B, Research Diets Inc.). While caution should be taken when applying findings to human research and health outcomes (e.g., anxiety, obesity, inflammatory conditions), some common themes that will be discussed can inform further clinical studies. As will be described below, in Study 1, I tested the impact of three rodent diets (i.e., WD D12079B, HCD D14042701, or SD 5001) on health-related parameters, whereas in Study 2, I administered two of these three diets (i.e., the WD or SD) with probiotic treatment (i.e., CEREBIOME® or placebo). Results from both studies can be compared to previous work from our lab (Myles, 2019; Myles, O'Leary, Smith, et al., 2020) where CEREBIOME® (or placebo) and WD D12079B (or the purified HCD control mentioned above) were administered to both sexes of Long–Evans rat. The collective results demonstrate how a specific probiotic strain combination and model of unhealthy diet interact to affect rat health-related outcomes, differentially in each sex (Figure 4.1).

4.1.1 Study 1 (Chapter 2)

The overarching goal of Study 1 (published as Myles et al., 2023) was to characterize certain psychological and physiological effects of WD administration compared to two of its commonly used control diets. Even though diet did not affect anxiety-related behaviour in the modified OFT on its own, males and females behaved differently with respect to exploration when placed into a novel apparatus in early adulthood (i.e., males performed more unsupported rears than females, females performed more head outs from a refuge area than males; see Figure 4.1). Furthermore, only in WD-fed animals did males perform more of the anxiety-related defensive behaviour, stretch attend postures, compared to females. While Study 1 did not include a stress manipulation *per se* (e.g., before

behavioural testing, including a non-stress exposed control group), exposure to a novel open field apparatus under white light during the dark phase of the light-dark cycle is an acute stressor and stretch attend postures are indicative of increased anxiety-related behaviour in that observational period (Dielenberg & McGregor, 2001; Kalueff & Tuohimaa, 2004). The frequency of stretch attend postures seems to be similarly affected by predator odour stress (i.e., increased) in male and female rats (i.e., Long–Evans exposed to cat collar in Kalynchuk et al., 2004; Sprague–Dawleys exposed to fox odour in Falconer & Galea, 2003). Although both studies report an increase in observed stretch attends with predator odour exposure, males, but not females, exposed to predator odour had specific hippocampal cellular changes (i.e., reduced cell death and reduced cell proliferation in the dentate gyrus; Falconer & Galea, 2003) and an increase in other defensive-related behaviours (i.e., head outs to the area with the predator odour; Kalynchuk et al., 2004). Even without a predator odour, stretch attend postures from a lower-anxiety area (i.e., closed section) to a higher-anxiety area (i.e., open section) are increased in the elevated zero maze in female C57BL/6 mice given WD (Bolton et al., 2017). In contrast, Wistar rats (male only) exposed to both comfort food and prior foot-shock stress displayed reduced anxiety-related symptomatology (i.e., more time in center of the OFT, decreased stretch attends and increased time in the open arm of the EPM), along with reduced serum CORT compared to control groups (Ortolani et al., 2011).

In general, these rodent studies suggest that stretch attend postures are occurring in response to aversive or anxiety-inducing situations. It is of interest that a sex difference of increased anxiety-related behaviour in males in Study 1 only occurs when rats are fed the WD. Of course, the interpretation of why unhealthy diets or predator odour might impact monitoring behaviours like stretch attends is complicated by the lack of studies that include both sexes. Interpretations are additionally perplexing because the stretch attend behaviour could differ in its presentation depending on the context of testing. For instance, while Bolton et al. (2017) are explicit in their description of where they are measuring stretch attends (i.e., from the lower anxiety area to the higher anxiety area), it is not clear how specific Ortolani et al. (2011) are in their measurement of stretch attends (i.e., in any area or only from closed to open arms). While predator odour exposure and approach-avoidant conflict-based tests are designed to elicit an anxiety-related presentation, exposing rodents

to the odour of a predator is a different circumstance than exposing them to the potential presence of a predator (i.e., an open area of approach-avoidant behavioural assays would increase the potential for predation; Lezak et al., 2017). As such, stretch attends during a predator odour exposure (i.e., towards the odour) could be a distinct behaviour from what is observed during the EPM, elevated zero maze, or OFT (e.g., stretch attends may be more akin to a head out if only measured at the threshold of anxiety-inducing areas).

Results from Study 1 establish that the type of administered experimental or control diet can differentially impact metabolic health. Specifically, regardless of sex, rats fed the WD and HCD demonstrate some metabolic similarities (i.e., weight gain, plasma leptin levels), but, of interest, HCD and SD rats were not found to differ in other measures of metabolic functioning compared to WD rats (i.e., both HCD-fed and SD-fed rats consumed fewer calories than WD-fed rats). Taken together with the finding of increased stretch attends in males compared to females fed the WD, previous work suggests that male rodents are quicker to show and are more severely affected by the negative behavioural and physiological impacts of WD or HFD administration when directly compared to females (R. B. S. Harris et al., 2003; Maric et al., 2022; Taraschenko et al., 2011). Increased levels of estradiol in females are often discussed as a potential influence for the protection from behavioural (e.g., improvements in hippocampal-dependent learning and memory in Frick et al., 2018) or metabolic (e.g., treatment with 17β-estradiol reduced HFD intake in Acharya et al., 2023) dysfunction after unhealthy diet exposure. Similar findings have also been described in humans; for instance, protection from cardiovascular disease has been reported in pre-menopausal females who would have higher estradiol levels than post-menopausal females and males (reviewed by Blenck et al., 2016).

Levels of estrogen receptors (e.g., ERα, ERβ) have also been implicated in mediating anxiety-related behaviour, in part via interactions with the HPA axis, with ERα often being deemed as anxiogenic (e.g., increases CRF) and ERβ as anxiolytic (e.g., reduces CORT; reviewed by Borrow & Handa, 2017). In females, levels of perceived psychological stress do not seem to correspond to levels of salivary or plasma cortisol (Bani-Issa et al., 2020; Kelly et al., 2008) and it has been suggested that CRF might be a more relevant marker of stress-associated anxiety in females (reviewed by Donner & Lowry, 2013). This theory is

of interest considering the co-morbid link between anxiety and obesity-related disorders in both animal models and humans. Indeed, CRF is critical in perpetuating the HPA axis response to stress (S. M. Smith & Vale, 2006) and is known for its anxiogenic effects, but CRF also interacts with leptin and NPY as an anorexigenic factor (Charmandari et al., 2005). Overall, the literature is lacking in animal and human studies with females, especially in research that directly compares females and males in the same study. Because of this gap in our knowledge of female physiology and behaviour, it is difficult to ascertain if specific methods (e.g., the interpretation of behavioural assay measures) and findings found in males (e.g., male Wistar rats exposed to stress and comfort foods display reduced anxiety-related behaviour; Ortolani et al., 2011) translate in any meaningful way to females until more research with both sexes is conducted.

4.1.2 Study 2 (Chapter 3)

With a continued focus on sex differences (see Figure 4.1 for an overview), the purpose of Study 2 was to characterize the impact of both WD (compared to SD) and probiotic treatment with CEREBIOME® (compared to placebo) in male and female Long–Evans rats. By incorporating the additional nutritional manipulation, CEREBIOME® treatment, results of Study 2 demonstrate the interactive effects of probiotic treatment, diet administration, and sex of rodent on physiological and behavioural outcomes. In complement to findings in Study 1, sex-specific responses to novel open field apparatus exposure (during the NSFT) are presented (e.g., females are more locomotive than males in the open field, neither sex differed in the amount of food eaten until feeding was analyzed in a familiar home cage observation). Analysis of adrenal gene expression also demonstrated that female rats have increased GR mRNA in their adrenal glands compared to males. This sex-specific finding warrants further study considering previous reports that there are physiological differences in the function and structure of male and female adrenal glands (Grabek et al., 2019; Ludescher et al., 2007) and because of the importance of adrenal gland function in disease development (Briassoulis et al., 2011; Munck et al., 1984; Tacon et al., 2009).

In complement to behavioural findings that probiotic animals display reduced anxiety-

related behaviour (i.e., more time spent in center) and are more inclined to eat in an open field apparatus after food deprivation, probiotic animals had higher NPY mRNA levels in their adrenal glands than placebo animals. Considering the documented links between the systems that regulate feeding and anxiety-related behaviour in the hypothalamus and the brainstem (Belgardt & Brüning, 2010; Könner & Brüning, 2012; Thaler et al., 2010), the finding of both increased adrenal NPY expression, reduced anxiety-related behaviour, and increased motivation to feed in CEREBIOME®-treated animals is interesting. What is of further interest is that increased NPY and NPY expression (most often measured or administered centrally) is known to be orexigenic (e.g., Kuo et al., 2007; Raposinho et al., 2001). However, probiotic animals of both sexes had a normalization of daily calorie intake when given the WD, whereas placebo rats fed the WD had increased calorie intake compared to their SD-fed counterparts. Relatedly, when examining correlations between NPY expression and feeding by experimental group, adrenal NPY expression at sacrifice was not found to be correlated with daily calorie intake in any group, but it was negatively correlated with calories consumed in the NSFT only in probiotic-treated, SD-fed males.

To compare to previous work from our group, Study 2 in this dissertation and Myles, O'Leary, Smith, et al. (2020) used the same experimental group design, similar experimental procedures, the same rat model, similar age of rats, probiotic (i.e., strains, dose, placebo), and WD. One major difference in the findings of the two studies that warrants discussion is the effect of CEREBIOME® treatment on inflammatory cytokine levels. In Myles, O'Leary, Smith, et al. (2020), the probiotic animals had increased levels of various plasma cytokines compared to placebo animals, whereas Study 2 reported increased hypothalamic cytokine levels in placebo rats compared to probiotic rats. The same Bio-Rad Pro^{TM} Rat Cytokine assay was used in both studies, but cytokines were measured in different sample types and the rats in Myles, O'Leary, Smith, et al. (2020) were exposed to a predator odour stressor (cat urine) before sacrifice. One explanation for these differential findings could lie in the predator odour stress exposure and associated steroid hormone release. With a chronic stress paradigm (i.e., water avoidance stress for one hour per day for four days), Ait-Belgnaoui et al. (2018) reported that two weeks of CEREBIOME® treatment (1×10^9 CFU per day) prevented the increase in stress-associated hormones (i.e., plasma CORT, noradrenaline, adrenaline) and increased GR

mRNA expression (i.e., in the hypothalamus, hippocampus, and prefrontal cortex) that was reduced in vehicle-treated rats exposed to stress. As such, it is possible that the predator odour stress increased the HPA axis response to a greater extent in placebo rats in Myles, O'Leary, Smith, et al. (2020). Considering the anti-inflammatory properties of glucocorticoids (McEwen et al., 1986; Munck et al., 1984; van der Velden, 1998), if placebo rats had an increased HPA axis response (e.g., released more of the glucocorticoid CORT) to the predator odour stress compared to probiotic rats (as shown previously in response to water avoidance stress by Ait-Belgnaoui et al., 2018), then this could help explain why probiotic rats had increased plasma cytokines compared to placebo animals after stress. Indeed, measuring stress-associated hormone and cytokine levels both centrally and peripherally in a future study would be beneficial for elucidating these disparate findings reported in Myles, O'Leary, Smith, et al. (2020) and Study 2.

Another experimental difference warranting discussion is the housing conditions of offspring rats during the lactation period in these two studies. Enhanced semi-naturalistic housing was used in Myles, O'Leary, Smith, et al. (2020), whereas mothers and their offspring were housed in standard housing in Study 2. Differences in housing during lactation have been shown to impact maternal care from mother rats and later offspring outcomes. With these same semi-naturalistic housing cages used during offspring rearing, Korgan et al. (2016, 2018) reported increased maternal care quality (i.e., more licking and grooming behaviour and improved nursing posture; see also Weaver et al., 2004) compared to standard-housed Long–Evans mothers. In addition, the male and female offspring from the semi-naturalistic cages had reduced presence of a marker of histone acetylation (H3K9ac) on the *Crf* promotor in the PVN (Korgan et al., 2016, 2018). Offspring from the semi-naturalistic housing rearing environment also spent more time in the center of the open field apparatus when tested in adolescence (Korgan et al., 2016). A later study with the stomachs from the rats of Korgan et al. (2018) determined that housing condition impacted the composition of the microbiota in this region (e.g., increased *Bifidobacterium pseudolongum* in the offspring reared in semi-naturalistic housing and who had sires given 60% HFD for 60 days prior to mating; Korgan et al., 2022). With a different model of enrichment in male and female Long–Evans rats (i.e., a social colony housing apparatus consisting of six interconnected standard housing cages and toys), Sparling et al. (2018)

demonstrated that adult offspring reared in enriched conditions had improved spatial memory and learning (tested in the Morris water maze). Recent work by Kentner et al. (2018) noted sex-specific changes in hippocampal expression of the CRF receptor 2 gene in that males exposed to six weeks of enrichment (i.e., multilevel cage with toys and four rats per cage) in early adulthood had increased hippocampal CRF receptor 2 expression while also spending less time in the light (LDB), while these differences were not observed in females. Evidently, enrichment during rearing or beyond can result in behavioural and physiological alterations and are one factor that may explain differences reported by Myles, O'Leary, Smith, et al. (2020) and Study 2.

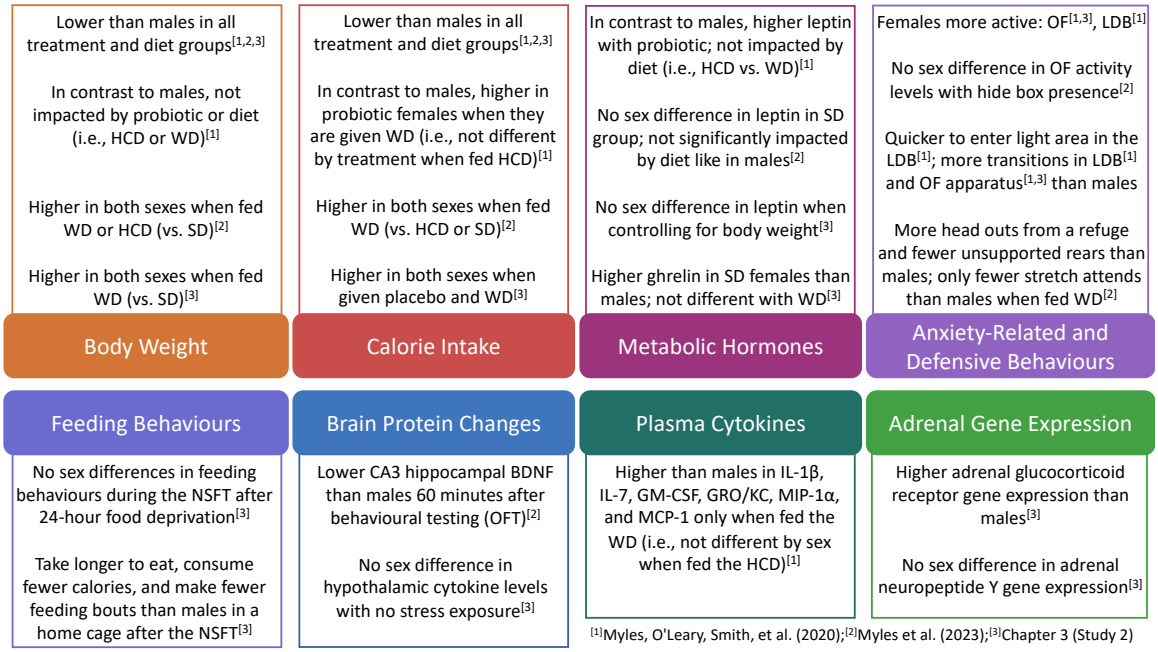

Figure 4.1 Sex differences (i.e., female relative to male) in study outcome measures from Myles, O'Leary, Smith, et al. (2020), Myles et al. (2023; Study 1), and Chapter 3 (Study 2). WD = Western diet, HCD = high-carbohydrate (control) diet, SD = standard diet, LDB = light-dark box, OF(T) = open field (test), NSFT = novelty-suppressed feeding task, IL = interleukin, GM-CSF = granulocyte/macrophage colony-stimulating factor, GRO/KC = growth-related oncogene/keratinocyte chemoattractant, MIP = macrophage inflammatory protein, MCP = monocyte chemoattractant protein.

4.2 Differences in the Effects of Specific Manipulations

4.2.1 Strain-Specific Effects of Probiotics

Probiotics have strain-specific effects, meaning that the same species of microbe might have multiple genetically different strains with specific purposes and mechanisms of action (Gareau et al., 2010; Williams, 2010). One example of strain specificity is the fact that *E. coli* strain Nissle 1917 is a non-pathogenic strain of *E. coli* that has been shown to protect against pathogenic bacteria (e.g., *Listeria monocytogenes*) and yeast (e.g., *Candida albicans*; an opportunistic pathogen) due to immunostimulant properties, whereby it can enhance immune responding to infection without being pathogenic itself (Hockertz, 1997). The strain *E. Coli* Nissle 1917 was first isolated from a specific German soldier's feces during World War I, who did not seem to develop symptoms of shigella infection, which was prevalent in many soldiers at the time, and it was demonstrated to inhibit growth against pathogenic Enterobacteriaceae (e.g., salmonella, listeria, shigella; Nissle, 1925, as reviewed in Sonnenborn, 2016). More generally, many strains of *E. coli* are non-pathogenic and are commensal bacteria that co-exist with hosts, but strain O157:H7 and certain other strains (e.g., eaggEC, enteroaggregative *E. coli*) can be life-threatening pathogens (Tarr, 1995). Of course, a critical factor in a specific strain of microbe being pathogenic, non-pathogenic, or beneficial is immune system responding and functioning of the host (e.g., people with HIV and low CD4 counts are highly susceptible to diarrheal illnesses from pathogens such as EaggEC; Bushen & Guerrant, 2008).

With the idea that probiotic formulations have strain-specific effects, probiotics are commonly comprised of a combination of strains that have been studied together, in isolation, and at varying dosages. For instance, *L. helveticus* strain R0052 is one type of *L. helveticus,* and behavioural and physiological effects could vary if another strain of *L. helveticus* is tested or if *L. helveticus* R0052 is combined with other probiotic strains (e.g., with 95% *Lacticaseibacillus rhamnosus* in Lacidofil®, with 10% *B. longum* in CEREBIOME®). Even though Lacidofil® and CEREBIOME® both contain *L. helveticus* R0052, Lacidofil® is studied for its documented benefits on aiding with stress coping and gastrointestinal dysfunction, whereas CEREBIOME® is commonly studied in models of

psychological distress or depression and associated gastrointestinal symptoms. Furthermore, one study that examined the CEREBIOME® combination of strains in comparison to *L. helveticus* R0052 or *B. longum* R0175 in isolation reported significantly increased benefits with the combination of strains (e.g., greater reduction in plasma CORT following stress with the CEREBIOME® combination; Ait-Belgnaoui et al., 2018).

A recently published systematic review and meta-analysis by McFarland and colleagues (2018) tested probiotic strain-specificity (i.e., 15 single-strain probiotics, 10 combination-strain probiotics) and documented the differential health-related effects of probiotic strains from the same genus and species (e.g., Lacidofil® has good support for *Helicobacter pylori* elimination, *Lacticaseibacillus rhamnosus* GG for alleviating symptoms of irritable bowel syndrome; McFarland et al., 2018). Interestingly, a recent meta-analysis reported that people with irritable bowel syndrome have an increased risk of *Helicobacter pylori* infection (Chunmei Wang et al., 2023), so further research on the effects of the Lacidofil® probiotic combination and *Lacticaseibacillus rhamnosus* GG on both irritable bowel syndrome and concurrent *Helicobacter pylori* infection could be warranted. Even if a probiotic strain does not have research support for a health-related benefit (e.g., CEREBIOME® is not studied for its effects on *Helicobacter pylori*), it does not mean that that probiotic could not impact that outcome. However, considering the stringent definition of a probiotic (see Section 1.1.7) provided by C. Hill and colleagues (2014), a probiotic must have a studied health benefit to be deemed as such. CEREBIOME® could have uncharacterized health-improving qualities, but these would need to be characterized in pre-clinical and clinical research at a specific dosage for probiotic benefits to be claimed.

Section 1.5.3 of this dissertation provides a more detailed account of the animal and human studies that have tested the effects of the CEREBIOME® probiotic formulation. However, it can be broadly summarized from the available research that CEREBIOME® treatment to male rodents at a dosage of 1×10^9 CFU per day reduces anxiety-related behaviours, HPA axis responding, gut barrier dysfunction, and cytokine release (Ait-Belgnaoui et al., 2014, 2018; Arseneault-Bréard et al., 2012; Girard et al., 2009; Messaoudi, Lalonde, et al., 2011; Mohammadi, Dargahi, Naserpour, et al., 2019; Mohammadi, Dargahi, Peymani, et al., 2019; Tillmann et al., 2018, 2021). Recent work by our laboratory group (Myles, 2019;

Myles, O'Leary, Smith, et al., 2020; Chapter 3 of this dissertation) extends this research to female rodents by providing preliminary evidence that CEREBIOME®-treated rats have reduced anxiety-related behaviours in the LDB and NSFT (e.g., increased motivation to feed in the NSFT), and results in lower hypothalamic cytokine levels compared to placebo rats. In complement, results outlined in Chapter 3 show a significant increase in the NPY gene in the adrenal glands, which, as a protein, is both anxiolytic and orexigenic. In humans, results from pilot and double-blind placebo controlled trials that include both male and female participants suggest that CEREBIOME® can impact psychological distress (namely depressive symptoms) and gastrointestinal disturbances, but that individual subject characteristics (e.g., baseline gut microbiota, baseline health status, sleep quality, physical activity) could be impacting the effects observed (Diop et al., 2008; Heidarzadeh-Rad et al., 2020; Kazemi et al., 2019; Messaoudi, Lalonde, et al., 2011; Messaoudi, Violle, et al., 2011; Morales-Torres et al., 2023; C. J. K. Wallace & Milev, 2021). Since these clinical studies already include both males and females and participant characteristics seem to be impacting the effects of CEREBIOME®, a more detailed investigation of participant characteristics (e.g., sex and gender) would be of interest.

4.2.2 Nutrients-Specific Effects of Diet

In their detailed review of preclinical WD use in research, Hintze et al. (2018) commented on the importance of diet-induced obesity models for human metabolic syndrome, obesity, and T2DM but stressed that generalizability to human health and disease is hindered by the study-to-study variation in types of diets. For instance, experimental diets (e.g., HFDs, WDs) can vary by more than just their macronutrient energy breakdown. Both the types of macronutrients (e.g., complex vs. simple carbohydrates, saturated vs. monounsaturated fats) and micronutrients (e.g., vitamin and mineral amounts, levels of electrolytes like sodium and potassium) can be drastically different even in diets with comparable macronutrient breakdowns (Hintze et al., 2018). This variability extends to preclinical studies that administer standard rodent diets without attempting to induce obesity as well. In fact, a recent study by Tuck et al. (2020) administered three commercially available SDs to male and female C57BL/6 mice (i.e., LabDiet® 5066, Research Diets AIN93G,

LabDiet® 5001) and reported striking differences in the cecal microbiota composition and microbiota metabolite presence (e.g., SCFAs).

One source of variation between rodent diets is the phytoestrogen level, of which soy products are a major source. Soy-based phytoestrogens have been reported to have both health-benefiting (e.g., lowering risk of heart disease) and health-harming properties (e.g., excessive menstrual bleeding; Patisaul & Jefferson, 2010). It is interesting that the results presented in Study 2 demonstrate reduced anxiety-related behaviour (i.e., more time in the center of the testing apparatus) specifically in CEREBIOME®-treated and SD-fed males. In fact, previous work from our laboratory group has not found that CEREBIOME® treatment, combined with WD or HCD, impacts time spent in the more aversive areas of behavioural testing apparatuses (i.e., the light section of the LDB, the center of the traditional OFT; Myles, O'Leary, Smith, et al., 2020). Similarly, providing an SD, along with the same WD and HCD was not found to impact time spent in the center of an open field with a refuge area (Myles et al., 2023). Interestingly, it has been reported that *B. longum* R0175 cultures grow better in soy-based beverages than in milk, whereas growth of R0052 was promoted in either milk or soy products (C. P. Champagne et al., 2009). Considering more recent reports that lactic acid probiotics might use dietary phytoestrogens to produce host-benefiting metabolites with estrogen-related activity (e.g., equol, enterolignans, urolithins; Landete et al., 2017), it is possible that the combination of CEREBIOME® and a soy-based diet with phytoestrogens is interacting to reduce anxiety-related behaviour in the males in Study 2. Since we are the only group to study the impact of CEREBIOME® treatment and WD in combination and Study 2 was the only time SD was administered to control for the WD, this theory would require further investigation before more detailed conclusions can be made. Further work could measure estrogen levels in experimental rats to see if levels vary by diet, phytoestrogens in SD pellets throughout the experiment, or microbiota-associated metabolites linked to phytoestrogens.

Over and above the intended nutritional composition of animal diets are diet components that are unintended or uncharacterized. Indeed, contaminants and environmental toxicants are present throughout the planet's ecosystem, and although much less of a concern for purified rodent diets, all rodent diets are comprised of agricultural products that are

vulnerable to contamination. As overviewed by Kumar and colleagues (2019), agricultural products can be contaminated by substances like pesticides (e.g., Roundup®), heavy metals (e.g., lead, cadmium, mercury), and organic pollutants (e.g., polycyclic aromatic hydrocarbons, polychlorinated biphenyls). Cereal products (e.g., wheat, corn) have also been reported to be contaminated by mycotoxins (i.e., toxic metabolites produced by fungi; e.g., aflatoxins), which, if levels are not properly monitored and exceed acceptable amounts, can cause organ damage and cancer (Luo et al., 2021).

Incidentally, probiotics are starting to be used for biological detoxification of mycotoxins to reduce their growth and reduce the absorption by human and animal guts (e.g., yeasts like *Saccharomyces boulardii*, lactic acid bacteria like *B. longum*; Emadi et al., 2022). Mesnage et al. (2015) conducted a study where they tested rodent SDs (e.g., Purina LabDiet® 5002 from Indiana, USA; SAFE A04 from France) obtained from suppliers from six continents (i.e., excluding Antarctica) for contaminants (e.g., pesticides, heavy metals). Results show that pesticide residues were present in all diets, although levels deemed hazardous varied based on diet type. Other contaminants were also present in varying amounts based on the diet (e.g., Purina LabDiet® 5002 chow had high levels of cadmium, the SAFE A04 chow was highest in arsenic; Mesnage et al., 2015). Trace or undetectable amounts of heavy metals and other pollutants like mycotoxins have been reported for purified diets (Pellizzon & Ricci, 2020), but it would be of interest to run a more detailed toxicological comparison of purified and non-purified diets in the same study and to analyze levels of toxicants during diet use as storage conditions can vary facility-to-facility.

4.3 Limitations of this Research

Despite some limitations of Study 1 (Chapter 2) and Study 2 (Chapter 3) being discussed within the discussion sections of those studies (i.e., Section 2.5 and 3.5, respectively), some limitations can be broadly applied to both studies and are reiterated as follows. First, only one behavioural test was conducted in each study to measure anxiety-, defensive-, and feeding-related behaviours. Due to not wanting to subject rats to multiple rounds of testing considering the repeated acute stressor, but also that repeated testing reduces behavioural responses over time (e.g., from lack of novelty, comfort with human contact and cage

transport; Hånell & Marklund, 2014), it was decided to increase the number of behaviours measured in one test. Compelling arguments can be made for either approach to behavioural testing, but one detailed behavioural test was chosen in these present studies as our goal was to garner a comprehensive picture of the anxiety-related behavioural state of the animals upon their first exposure to a novel paradigm. The results of Study 2 can be complemented by findings from Myles, O'Leary, Smith, et al. (2020) where CEREBIOME®-treated rats made more transitions between the light and dark section of the LDB apparatus than placebo rats, with no difference in overall distance travelled (measured by manual scoring of line crosses in the apparatus). However, it should be acknowledged that measuring behaviours that are rarely measured and modifying traditional and well-tested behavioural testing apparatuses (e.g., adding a hide box and adjusting the center area of the OFT in Study 1) limits the generalizability of our findings.

Considering the limited metabolic-related findings in Study 1, especially for females, with only six weeks of diet administration and only five weeks of weight gain available to measure before subjecting rats to behavioural testing (see Diane et al., 2008 for an example of how acute stress can modify feeding amounts and preferences differentially in male and female rats), it was the goal to extend probiotic treatment and diet administration in Study 2. However, due to COVID-19-related factors and necessary disruptive construction in our animal care facility, Study 2 was limited to seven weeks of both probiotic and diet administration. Coincidentally, the timeline for Study 2 ended up being nearly identical to Myles, O'Leary, Smith, et al. (2020), which allowed for the provision of a more comparable, albeit not identical (see Section 4.1), reproduction of our previous findings as Long–Evans rats were of similar age and provided with experimental manipulations for the same time frame of seven weeks.

In an ideal situation where resources and experimenters were not limited by extraneous factors (e.g., ability to be trained on live blood drawing techniques, COVID-19-related personnel capacity limitations in the facility), both Study 1 and Study 2 could have benefited from additional metabolic-related measures including insulin and glucose tolerance studies. Along these same lines, measuring additional steroid hormones such as sex hormones (e.g., estradiol, testosterone) and HPA axis hormones (e.g., CORT, CRF)

could provide additional insight into the mechanisms behind the sex-specific alterations in behaviour and hormonal response to CEREBIOME® treatment and WD. Indeed, increased estradiol in female rodents is linked to increased CORT release at baseline or when exposed to stress, whereas increased androgens can result in blunted CORT and ACTH release with stress (reviewed by Oyola & Handa, 2017). Moreover, gonadectomy in female and male rats seems to remove the sex difference in HPA axis functionality with stress exposure (i.e., castrated males resemble gonadally intact females; ovariectomized females resemble gonadally intact males; Seale et al., 2004). In humans, both anxiety symptoms and obesity have been found to be positively correlated with testosterone levels in premenopausal women (Stanikova et al., 2019), highlighting the importance of considering sex hormone levels in anxiety and obesity development and maintenance.

One experimental paradigm that was optimized and shown to be logistically possible, even with the COVID-19 pandemic, was a gastric emptying paradigm before sacrifice. Studying alterations in gastric emptying is directly linked to food intake and could help inform the etiology and treatment success for obesity and associated conditions (Goyal et al., 2019). More specifically, ghrelin, leptin, and NPY have all been shown to affect gastric emptying, with ghrelin being associated with increased motility (Müller et al., 2015), and leptin, NPY, GLP-1, and cholecystokinin with reduced motility (Goyal et al., 2019; Ishiguchi et al., 2001; Smiley et al., 2020). However, as will be explained, optimization of this protocol with practice female rats, revealed a critical shortcoming that could have impacted study findings had this published protocol (Asakawa et al., 1999; Asakawa, Inui, Kaga, Yuzuriha, Nagata, Ueno, et al., 2001) not been verified in the present sample of rats. Briefly, the protocol involves food deprivation for 16 hours to completely empty the stomach, reintroducing a pre-weighed amount of food for exactly two hours, sacrificing the rat as normal, and weighing the amount of food recovered from the stomach to calculate how much had emptied. With SD-fed practice rats, it was first verified that a 16-hour fast was sufficient to empty the stomach in female Long–Evans rats as the work from Asakawa et al. (1999) and Asakawa, Inui, Kaga, Yuzuriha, Nagata, Ueno, et al. (2001) used male mice of different rodent strains. It was found that a 16-hour fast was sufficient to empty the stomach, and food was present in the stomach after a two-hour reintroduction and easily calculated from the weight of stomach contents.

Unfortunately, once this protocol was attempted in female rats who had been administered a WD, it was found that the stomachs of these rats contained some softwood bedding from their cages and, thus, it was not possible to calculate how much food remained. Although the CCAC has reported that consumption of feces or bedding could occur for fasts that are 24 hours or longer (Canadian Council on Animal Care, 2020), this finding after 16 hours was unexpected because it did not occur in any SD-fed practice rats, and the bedding used was not food based like other bedding types (e.g., corn cob bedding). Fasts up to 24 hours are reported in research studies and within the CCAC guidelines, but a 16-hour fast is reported to be preferred to a 24-hour fast when possible because it does not result in increased CORT compared to controls (Nowland et al., 2011), although both types of fasts are common in research studies. However, our findings from optimization suggest that WD-fed rats seem to be more impacted or quicker to be impacted by a fast, which could be because they are attempting to fill their stomachs with something as they want to engage in feeding behaviours even if the substance is not food-based. This finding that WD-fed rats could be more impacted by fasting warrants consideration in diet-induced obesity studies that include a fasting protocol.

Our next steps with this research would be to expand on our findings by studying the composition of the microbiota and correlating levels of known taxa to physiological changes (e.g., NPY expression). Although such results would be beyond the scope of this dissertation, because caecal content samples were collected for the confirmation of probiotic strain presence (or absence), some of this DNA was normalized for sequencing of the V3 – V4 region of the bacterial 16S ribosomal RNA gene (Illumina MiSeq™ System, cat. #M-GL-00006). The bacteria that reside in specific areas of the gut can be characterized by sequencing this 16S gene (Rinninella et al., 2019). Briefly, in duplicate, for all mother rats and offspring samples, two PCR reactions were conducted to amplify the 16S gene (i.e., to ensure enough copies are present, flank the region of interest on the gene, and add index sequences and adaptors for the Illumina system to read), with associated clean-ups along the way. Then, sample DNA was sequenced with the MiSeq system to characterize the bacterial groups that are present. This data can be used in the future (i.e., with bioinformatic processing) to describe the composition of the gut microbiota by sample or group. Indeed, furthering our understanding of the specific

microbes that comprise the gut microbiota after experimental manipulations can provide insight into why such manipulations might change behaviour and physiology. Besides describing which taxa are present in the microbiota, the relative abundance of those taxa can be compared across different subjects, and diversity metrics (e.g., alpha diversity, beta diversity) can be calculated (Cryan et al., 2019). Further, when characterizing which types of bacteria are present in the gut, a next step for research will be describing the function of these bacteria through the identification of the metabolites they produce, as these metabolites have been reported to mediate observed host benefits (O'Connell, 2020; Spacova et al., 2020).

4.4 Potential Targets for Further Study

4.4.1 Probiotic Mechanisms

The development of the gut microbiota (e.g., composition, diversity) has been associated with antibiotic exposure, delivery type (Caesarean section vs. vaginal), and diet (formula vs. breast-feeding; Bokulich et al., 2016; Dominguez-Bello et al., 2010). Previous work has reported differences in the risk of later diseases such as allergies and T2DM in formula- compared to breast-fed infants (Owen et al., 2006; Przyrembel, 2012). Interestingly, the feces of breast-fed infants contains more of the beneficial bacterial genus *Bifidobacterium*, whereas the feces of formula-fed infants contains more of the genus *Atopobium* (Bezirtzoglou et al., 2011), which is a genus with species linked to bacterial vaginosis infections (Mendling et al., 2019). In a clinical study that found improved psychological symptomatology and lower urinary-free cortisol after CEREBIOME® treatment, Messaoudi, Violle, et al. (2011) speculated that possible mechanisms for these findings could be a reduction in pro-inflammatory cytokines, increased neurotransmitter production, or increased commensal bifidobacteria presence in the gut from probiotic treatment. A recent study with small intestines and colons from C57BL/6J male mice showed that the function of enterochromaffin cells (that produce much of the body's serotonin) from these tissues is affected by gut microbiota and food metabolites (Lund et al., 2018). Although the complete mechanistic pathway has not been confirmed, it was

proposed that in the small intestine, GLP-1 secretion from GLP-1 cells binds to GLP-1 receptors on the enterochromaffin cells in response to food components and leads to the release of serotonin from enterochromaffin cells (Lund et al., 2018). In parallel, it is speculated that gut microbiota metabolites in the colon affect serotonin release from the enterochromaffin cells in this region and result in serotonin production that also affects other systems in the body (e.g., vagal nerve transmissions; Lund et al., 2018).

As touched on in Chapter 1, probiotics are commonly comprised of specific bacterial strains, sometimes in combination with yeast, but other types of probiotics are being studied for their health-promoting effects (e.g., exclusive yeast probiotics, archaea probiotics). For instance, *Saccharomyces cerevisiae boulardii* has been reported to aid with alleviating gastrointestinal infection, but even on its own, this yeast (a common 'nutritional yeast') is a protein source that has antioxidant properties (e.g., can produce glutathione) and contains important vitamins and minerals (e.g., B vitamins, iron; Abid et al., 2022). Recently, archaea probiotics have been theorized to have therapeutic use in humans due to their non-pathogenic nature and the fact that species of archaea are already present as an, albeit minor, part of the human gut microbiota (Brugère et al., 2018). For instance, Fadhlaoui et al. (2020) review the idea that specific species of archaea could use harmful bioproducts of human diets (e.g., trimethylamine oxide from consuming red meat) as part of their own metabolism and help humans more safely excrete these compounds (see also Ramezani et al., 2018). Additionally, archaea of the order Methanobacteriales (who produce methane) have been reported to be increased in people with obesity compared to 'normal'-weight individuals and who have undergone gastric-bypass surgery (H. Zhang et al., 2009), so researching the health-related implications of this finding (e.g., are these bacteria there as a cause or a consequence of obesity) could prove to be important in the study of the development of obesity. As a whole, non-bacterial probiotic strains seem uniquely qualified to alleviate specific health concerns and warrant continued investigation to further elucidate the mechanisms responsible for their benefits.

4.4.2 The Hypothalamic-Pituitary-Adrenal Axis

The HPA axis can be regulated at various stages, independent of glucocorticoids. For

example, CRF binding protein can inhibit CRF and the release of ACTH (Linton et al., 1993; Westphal & Seasholtz, 2006). Of interest, a recent study reports on preventing the release of ACTH by inhibiting CRF with the monoclonal antibody CTRND05 (i.e., an antibody that binds to and inhibits CRF in multiple strains of mice), which has implications for various types of disease states in humans (e.g., neuropsychiatric, metabolic; Futch et al., 2019). As touched on in Section 4.3, incorporating additional markers of HPA axis functionality (e.g., CRF, ACTH) at different time points or with previous stress exposure could provide valuable information as to why specific probiotic strains affect psychological functioning in both human and animal studies.

GR expression is a crucial consideration in the etiology of stress- and anxiety-related symptomatology. For instance, early-life stress reduces GR mRNA and is associated with anxiety-related behaviour in male mice (Arnett et al., 2015). Further, knockout mice with disrupted GR in the hippocampus and cortex (FBGRKO) show an unexpected response to the LDB (i.e., they enter the light earlier than controls after being exposed to stress, whereas controls have delayed entry after stress; Boyle et al., 2006). A recent study with male and female C57BL/6 mice found that administering CORT induces depressive- and anxiety-related behaviours (L. Lin et al., 2022). As well, CORT administration increases proBDNF (a BDNF precursor) in the hippocampus and cerebellum, increases both proBDNF and mature BDNF in the pituitary, but decreases protein levels of both in the adrenal gland (L. Lin et al., 2022). In another study, administering the synthetic glucocorticoid dexamethasone (a GR antagonist) to pregnant Swiss mice (in order to harvest fetal mouse hippocampal neurons) downregulated *Bdnf* expression by GR binding to *Bdnf* promotor regions (H. Chen et al., 2017).

4.4.3 Brain-Derived Neurotrophic Factor

As previously described, reduced BDNF has been linked to stress exposure and anxiety-related behaviour in rodent models (reviewed by Duman & Monteggia, 2006; Murínová et al., 2017, respectively) and anxiety- and stress-related disorders in humans (Dell'Osso et al., 2009; I. M. dos Santos et al., 2011; Ströhle et al., 2010). As well, rodent work has reported decreased hippocampal *Bdnf* expression with exposure to unhealthy diets (i.e.,

WD in Molteni et al., 2002; 60% HFD in N. Yamada et al., 2011; HCD in Maioli et al., 2012) and increased hippocampal BDNF after leptin administration (i.e., BDNF mRNA in C. Li et al., 2021; protein in N. Yamada et al., 2011). BDNF levels are also impacted by probiotic treatment; early work in this area (see Section 1.2.6) reported reduced *Bdnf* expression and BDNF protein levels in the cortex and hippocampus of germ-free animals compared to specific pathogen-free mice (male BALB/c; Sudo et al., 2004).

One noteworthy area of probiotic research that relates to BDNF is studying how these microbial strains affect vagal nerve transmissions between the brainstem and periphery and modulate brain physiology and associated behaviours (Forsythe et al., 2014). With the knowledge that probiotic bacteria can increase *Bdnf* expression in the hippocampus and the hypothesis that these effects are regulated via the vagus nerve, O. F. O'Leary and colleagues (2018) determined that vagotomy in mice decreased *Bdnf* in the hippocampus. Further, with a model of male AKR mice infected with parasitic *Trichuris muris*, Bercik et al. (2010) showed that treatment with *B. longum* NCC3001 normalized anxiety-related behaviour (e.g., time in light in the LDB) and increased CA1 hippocampal BDNF mRNA that was reduced by infection. However, in the latency to step-down test, mice infected with *Trichuris muris* showed increased anxiety (i.e., increased time to step down from an elevated platform) compared to control mice, whether or not they were vagotomized (Bercik et al., 2010).

Evidently, communication between the brain and periphery and the associated effects on physiology and behaviour, whether that be dependent or independent of the vagus nerve, is an important topic for further study. In the previously mentioned study by Gareau et al. (2011; see Section 1.1.4), Lacidofil® treatment was shown to restore BDNF (by immunohistochemistry) in the CA1 region of the hippocampus that was reduced with *Citrobacter rodentium* infection in female C57BL/6 mice. It has also been reported that *B. longum infantis* CCFM687 supplementation increases BDNF protein levels in the prefrontal cortex and reduces certain anxiety-related behaviours (e.g., increases time spent in the open arms of the EPM) following a chronic stress paradigm in adult C57BL/6J mice (no sex reported; Tian et al., 2019). In this work, chronic stress was also shown to increase HPA axis activity (i.e., increase hypothalamic CRF, serum ACTH, serum CORT), and this

increased HPA axis activity was reduced with both the antidepressant fluoxetine and the probiotic (Tian et al., 2019). The probiotic was also able to reverse the stress-induced increase in serum TNF-α, whereas fluoxetine had no effect (Tian et al., 2019). Taken together, measuring behavioural and physiological changes in BDNF in different brain regions in response to experimental manipulations like diet, probiotic treatment, or stress exposure could provide additional insight into the potential interactive health effects of these variables.

4.4.4 Ghrelin

As previously introduced in Sections 1.3.2 and 1.3.3, ghrelin is an orexigenic hormone that can be acylated or deacylated (Y. Wang et al., 2022). When acylated, ghrelin can bind to its receptor and has been demonstrated to induce food intake and affect stress- and anxiety-related responses in animal models and humans (Carlini et al., 2002; Jensen et al., 2016; Naufel et al., 2021; Schaeffer et al., 2013). However, as described in detail by Delhanty et al. (2014), new research is highlighting that deacylated ghrelin, which does not bind in any meaningful way to the ghrelin receptor, could have opposite functionality to acylated ghrelin and function with another receptor, which has implications for preclinical and clinical studies on obesity and related health complications. Interestingly, a recent systematic review and meta-analysis of human studies that measured acylated and deacylated levels in 'normal'-weight and adults with obesity revealed that both ghrelin isoforms are lower in people with obesity (Y. Wang et al., 2022).

Research into the physiological role of deacylated ghrelin is gaining traction, especially in people with T2DM, because this form of ghrelin could be important when studying prevention and treatment options for metabolic-associated disease states. For instance, deacylated ghrelin has been reported to be reduced in people with T2DM and obesity, with no change in acylated ghrelin in this group (Zang et al., 2022), and deacylated ghrelin has also been proposed to function, at least in part, as an acylated ghrelin antagonist (Fernandez et al., 2016). Overall, the inhibition of ghrelin signalling (i.e., circulating acetylated ghrelin) shows some promise in the treatment of metabolic syndrome, T2DM, and obesity (reviewed by Schalla & Stengel, 2019; see also Asakawa et al., 2003; Barnett et al., 2010;

Gagnon et al., 2015), but more research on ghrelin interference in humans is warranted, especially with respect to potential mental health-related side effects.

4.4.5 Short-Chain Fatty Acids

SCFAs are a group of metabolites (e.g., acetate, propionate, butyrate) that are produced by the gut microbiota during the digestion of complex carbohydrates (i.e., fibre, resistant starch) in the large intestine. Production of SCFAs in any physiologically relevant amount is presumed to not be possible without a gut microbiota as germ-free rodents have low concentrations of SCFAs in their digestive tracts, compared to conventional animals (assumed to come from the diet they eat; Høverstad & Midtvedt, 1986). Turnbaugh et al. (2006) showed that relative to lean mice, *ob*/*ob* C57BL/6J mice (no sex reported) have increased butyrate and acetate in their caeca, increased members of the Firmicutes phylum, and reduced energy stores (kcal/g) in their feces. Further, colonizing germ-free mice with caecal microbiota from the *ob*/*ob* mice, as compared to the lean mouse donors, significantly increased body fat of recipient mice (Turnbaugh et al., 2006).

Concerning butyrate, it is of interest that Turnbaugh et al. (2006) reported increased levels of SCFAs in the *ob*/*ob* mice, which they proposed might be a compensatory mechanism for dealing with increased available energy storage. It has been reported that SCFAs like butyrate could be beneficial in treating obesity and T2DM (e.g., reducing LPS levels, improving insulin resistance, increasing serotonin; reviewed by Hartstra et al., 2015). In a human study that included males and females, participants categorized as 'obese' by BMI were found to have higher concentrations of SCFAs in their feces compared to lean participants (Schwiertz et al., 2010). As well, supplementation of butyrate, acetate, and propionate to C57BL/6J male mice was found to inhibit body weight gain, triglycerides, and pro-inflammatory cytokines that were increased in response to HFD (60% kcal fat, 20% kcal carbohydrate; Lu et al., 2016). Presence of one butyrate-producing bacterial species, *Faecalibacterium prausnitzii,* of the Firmicutes phylum, seems to be important for the prevention and improvement of T2DM (reviewed by Ganesan et al., 2018). On top of the effects of SCFAs on both behaviour and metabolism, levels of SCFAs also appear to interact with dietary fibre in these relationships. For instance, one study reports that

increased abundance of the genus *Parabacteroides* is related to lower dietary fibre intake, unhealthy eating behaviours, and increased fecal SCFA presence (Medawar et al., 2021).

SCFAs have also been reported to reduce anxiety- and depressive-related behaviours. In male C57BL/6J mice, increased time spent in the center of the OFT and decreased immobility time in the forced swim test was observed after the administration of SCFAs (i.e., a mixture of acetate, propionate, and butyrate; van de Wouw et al., 2018). Although the SCFA treatment did not directly affect body weight, the chronically stressed rats that had increased body weight also had increased SCFAs in their feces (van de Wouw et al., 2018). Another study with male C57BL/6J mice administered an SCFA mixture after antibiotic treatment (or antibiotic vehicle control; J.-T. Wu et al., 2022). The antibiotic treatment resulted in decreased levels of acetate, propionate, and butyrate, along with reducing anxiety-related behaviours (e.g., increased center time in the OFT), whereas SCFA treatment increased anxiety-related behaviours, comparable to those of the vehicle-treated mice (J.-T. Wu et al., 2022). Surprisingly, these findings were not explained solely by vagus nerve communication, as increased anxiety-related behaviours after SCFA treatment in the antibiotic-treated mice still occurred after vagotomy (J.-T. Wu et al., 2022).

4.4.6 Epigenetic Changes

Weaver and colleagues (2004) demonstrated in male Long–Evans rats that good quality maternal care in the first postnatal week resulted in increased DNA acetylation and reduced DNA methylation at a specific region (NGFI-A binding site) on the GR gene promotor (i.e., in the exon 1_7 region). These epigenetic changes resulted in increased expression of the GR gene in the hippocampus of these offspring rats, which affected their stress responding in adulthood (i.e., plasma CORT was reduced after a restraint stressor; Weaver et al., 2004). Even in rats that received low-quality maternal care and had an increased HPA axis CORT response to the restraint stress, the administration of trichostatin A (an inhibitor of histone deacetylation processes) increased DNA acetylation, reduced DNA methylation at the exon 1_7 promotor region, and reduced the increased CORT response post-stress (Weaver et al., 2004). A follow-up study with male Long–Evans offspring of high- or low-quality maternal care showed that administering the amino acid, L-methionine

(which can provide methyl groups) increases DNA methylation at this same region in the GR gene (Weaver et al., 2005). In contrast to trichostatin A, L-methionine treatment increased CORT after restraint stress and increased immobility time in the forced swim test (i.e., a depressive-related behaviour; Weaver et al., 2005).

Alongside these findings from Weaver and colleagues (2005) that methionine can increase stress and depression-related behaviours, human diets deficient in methyl groups can also promote depression if levels are below a healthy threshold (Ullah, Khan, et al., 2022). In fact, in a placebo-controlled double-blind clinical trial, participants with depressive symptoms that did not reach the criteria for major depressive disorder were administered a combination psychobiotic (i.e., CEREBIOME® at 3×10^9 CFU/day and 200 mg S-adenosyl-L-methionine) for three months and treatment reduced symptoms of depression compared to placebo (Ullah, Di Minno, et al., 2022). However, both S-adenosyl-L-methionine (e.g., Sarris et al., 2020) and CEREBIOME® (e.g., Kazemi et al., 2019; Wallace & Milev, 2021) have been shown to improve depressive symptoms in humans, so it would be interesting for further work to examine both treatments in isolation and in combination in the same group of participants (e.g., with depressive symptoms, with a diagnosis of major depressive disorder).

In a recent study, C57BL/6 male mice were administered SD (Purina LabDiet® 5001) or 60% HFD (20% kcal carbohydrate) with additional sucrose in water from six weeks old until 14 months old and were additionally administered S-adenosyl-L-methionine for the remaining six weeks of the experiment (Vander Velden & Osborne, 2021). In this group of middle-aged mice, S-adenosyl-L-methionine treatment improved metabolic-related outcomes (e.g., reduced fasting glucose), but only in mice fed the SD. As well, the diet-induced obesity mice showed increased anxiety in the OFT (e.g., reduced time spent in center, increased latency to enter center), but S-adenosyl-L-methionine treatment only partially improved anxiety-related behaviour in this group (i.e., reduced latency to enter the center) and did not improve the already reduced anxiety-related symptomatology in the SD-fed mice (Vander Velden & Osborne, 2021). These studies provide an example of how probiotic treatment has the potential to interact with diet components or a diet that has an excess or deficiency in a certain ingredient, which highlights a broad but important area of

further study with respect to the health effects of probiotics in specific hosts.

4.5 Future Directions and Considerations

4.5.1 The Importance of Studying Both Male and Female Rodents

The exclusion of females from animal and human studies has been historically rationalized because of the concern that the estrous cycle would result in findings being too variable or difficult to interpret (reviewed by Beery & Zucker, 2011). However, a recent meta-analysis of over 300 neuroscience studies that included both male and female rats concluded that this concern for variability was unfounded because data were not more variable in females compared to males, even in studies that did not measure or control for estrous cycle phase (Becker et al., 2016). When examining specific subcategories of data, females did show more variability in blood and organ measures than males, but males showed increased variability in neurochemistry measures and electrophysiology measures (Becker et al., 2016). In the attempt to understand the etiology of stress-related, metabolic, or any other altered physiological or psychological function, disregarding one sex will continue to leave many questions unanswered. Even male and female rodents that are in the same study and exposed to the same manipulations do not respond to the experiment in similar ways, as exampled by previous findings (see Figure 4.1) by our laboratory group (Myles, O'Leary, Smith, et al., 2020; Myles et al., 2023; Chapter 3 of this dissertation) and the results of other research (Bridgewater et al., 2017; Esposito et al., 2022; Maniam & Morris, 2010; Org et al., 2016; Sasaki et al., 2014; M. Schell et al., 2023; St-Cyr, Abuaish, Welch, et al., 2018; St-Cyr et al., 2017; Sturman et al., 2018; Turner & Weaver, 1985; Warneke et al., 2014; Werdermann et al., 2021; Yang et al., 2014).

When both sexes are behaviourally tested in the same study, it has been consistently shown that female and male rodents are distinct in their response to various types of experimental manipulations (e.g., HFD and stress exposure in Bridgewater et al., 2017; prenatal stress in Panetta et al., 2017; WD and HCD in Myles et al., 2023; stress exposure in Weisbrod et al., 2019). For instance, Sturman et al. (2018) reported differences in the presentation of anxiety-related behaviour of adult male and female C57BL/6J mice. In contrast to males,

the behaviour of the female mice was not found to be altered when changing the level of background noise, but the presence of white light during the OFT led to greater distance travelled in the females (Sturman et al., 2018). Repeated testing also altered rearing frequency in females but not males (Sturman et al., 2018), which is an important sex-specific finding that should be considered in studies that employ repeated testing paradigms.

These sex-dependent findings are not limited to behavioural measures; rodent studies that include both sexes in their sample are consistently finding distinct molecular changes in response to experimental manipulations (e.g., diet exposure, early life stress exposure) as well. With both Sprague–Dawley rats and C57BL/6N mice fed HFD (60% kcal fat, 20% kcal carbohydrate) or an SD (Envigo 18% Protein diet), Maric et al. (2022) showed that female rats and mice had a greater preference for the HFD compared to males, and female rats had a delay in the onset of negative metabolic consequences of the HFD. Earlier work with male and female Sprague–Dawley rats (Taraschenko et al., 2011) and NIH Swiss mice (R. B. S. Harris et al., 2003) fed WDs compared to HCD controls and further report on the difficulty of inducing obesity in female rats, which is dependent on both the length of diet administration and age of the rodents when diet administration begins.

Related to the gut microbiota, adult ovariectomized female C57BL/6J mice treated with 17β-estradiol had reduced intake of an HFD (60% kcal fat, 20% kcal carbohydrate) and reduced anxiety-related behaviour in the LDB (Acharya et al., 2023). While the HFD resulted in changes to the gut microbiota compared to SD-fed mice (14% kcal fat, 47% kcal carbohydrate), 17β-estradiol treatment interacted with the poor diet to prevent some changes in taxa (in fecal samples) that were associated with anxiety-related behaviours (e.g., *Coprococcus* genus, Mogibacteraceae family; Acharya et al., 2023). In another recent study, with both male and female C57BL/6J mice fed HFD (60% kcal fat, 20% kcal carbohydrate) or HCD control (10% kcal fat, 70% kcal carbohydrate), Hases et al. (2023) reported sex-specific caecal microbiota compositions (e.g., increased *Blautia hansenii* in females compared to males). The authors note additional sex-specific interactions on the gut microbiota with HFD administration (e.g., increased abundance of three *Lachnospiraceae* spp. in females fed the HFD), along with taxa shifts in HFD males given

17β-estradiol (e.g., increased abundance of *Collinsella aerofaciens*; Hases et al., 2023).

In humans, a recent structural MRI imaging study determined that obesity (as measured by BMI) and major depressive disorder can result in similar structural brain abnormalities (e.g., reduced thickness of the temporal and frontal cortices), a relationship that seems to be more pronounced in female participants (Opel et al., 2021). In their review, Bekhbat and Neigh (2018) conclude that inflammation and stress-related psychological symptomatology are interrelated, and more so, in females compared to males. Furthermore, sex hormones (e.g., estradiol) affect the functioning of the HPA axis, which seems to, in turn, impact physiological functioning in a sex-specific manner and may be directly related to the adrenal glands. For instance, human males have been reported to have more visceral fat and less subcutaneous fat than females, but in female participants only, adrenal gland size has been found to be highly positively correlated with the amount of visceral fat (measured by whole-body MRI; Ludescher et al., 2007).

4.5.2 Treatment Options for Anxiety and Obesity

There are monogenic causes of obesity (e.g., autosomal recessive leptin deficiency or leptin receptor deficiency; summarized by Bouchard, 2021). However, most causes of obesity are due to combination genetic (e.g., SNPs as mentioned in Sections 1.1.2, 1.1.7, and 1.2.2; see also Cuevas-Sierra et al., 2020; Sovio et al., 2011) and environmental risk factors (e.g., sex, socioeconomic status, stress exposure; see Blüher, 2019 for a review). Historically, a CB1 cannabinoid receptor antagonist was developed (i.e., SR141716A; Rinaldi-Carmona et al., 1994) that was later reported to reduce food intake through interactions between leptin and endocannabinoids in the hypothalamus (Di Marzo et al., 2001). This compound, later developed as the drug Rimonabant in Europe, was highly successful, when combined with a reduced calorie diet, in lowering obesity presence and associated metabolic abnormalities (Gelfand & Cannon, 2006; Van Gaal et al., 2005), but with devastating consequences when it came to symptoms of anxiety, depression, and suicidal ideation (Di Marzo et al., 2001; Sam et al., 2011). Recently, semaglutide (i.e., a glucagon-like peptide-1 agonist that increases insulin secretion to treat T2DM) has gained popularity for its anti-obesity effects. Specifically, when it comes to the treatment of obesity, semaglutide reduces

hunger and increases feelings of fullness, which has been shown in clinical trials to significantly reduce weight (Bergmann et al., 2023) and blood pressure (C. Kennedy et al., 2023) even without T2DM presence. As future studies are conducted on the use of semaglutide, it will be of interest to report on the mental health effects of treatment, as there is a stark lack of available studies that include these parameters.

As exemplified by Rimonabant, obesity is extremely difficult to treat, and mental health and cardiac side effects are common, resulting in the use of various drugs no longer being justified (i.e., the risks outweigh the benefits; e.g., sibutramine; James et al., 2010). While there are currently approved pharmacological treatment options for obesity (e.g., semaglutide) and recommended lifestyle interventions, these methods can be expensive (e.g., cost of drugs like semaglutide, healthy foods) and do not work for all people (Blüher, 2019). As such, researching impacts on the gut microbiota provide another treatment avenue or prevention strategy for obesity and related complications. Indeed, the gut microbiota is directly linked to metabolic functioning (Arora et al., 2021; Everard et al., 2013; R. Gao et al., 2018; Trasande et al., 2013) and nutritional interventions designed to impact the functioning or composition of the microbiota (e.g., diet, probiotics) have shown promise for the prevention or treatment of obesity (Brahe et al., 2015; Everard et al., 2013; Z.-B. Wang et al., 2019; L. Zhao et al., 2018). Relatedly, fecal microbiota transfers have shown some success with respect to improving insulin sensitivity when transferred from lean subjects to people with obesity (Vrieze et al., 2012), as has the administration of SCFAs, which have been reported to regulate appetite (e.g., increase GLP-1) and improve gut barrier integrity (reviewed by Alhabeeb et al., 2021; see also Section 4.4.5).

Compared to obesity, there are many potential options for treating anxiety disorders in humans (e.g., selective serotonin reuptake inhibitors, serotonin norepinephrine reuptake inhibitors, benzodiazepines, anti-psychotics). In general, pharmacological interventions and cognitive behavioural therapy are most effective at treating anxiety disorders (Bartley et al., 2013). However, this effectiveness does not generalize to all people and there is a lack of research in studying novel treatments for anxiety disorders compared to other psychological disorders (e.g., major depressive disorder; Garakani et al., 2020). Specifically, it has been documented that just greater than half of people respond to the

traditional treatment options for anxiety disorders (i.e., selective serotonin reuptake inhibitors, cognitive behavioural therapy; Bystritsky, 2006), highlighting the need for further work in this area. For people who have treatment-resistant anxiety, non-pharmacological interventions have shown some promise, such as exercise (Kandola et al., 2018) or adding music interventions to psychotherapy (Shirani Bidabadi & Mehryar, 2015; Trimmer et al., 2018). A recent scoping review, including human and animal model studies, determined that specific dietary factors were found to reduce anxiety symptoms or the prevalence of anxiety disorders (e.g., calorie restriction, increasing fruits and vegetables, micronutrients such as zinc or magnesium, probiotics; Aucoin et al., 2021). Additionally, a systematic review and meta-analysis highlighted a small effect in the treatment of depression and anxiety with probiotics, with a larger effect with clinical subjects compared to community participants (R. T. Liu et al., 2019).

4.5.3 Individual Differences in Obesity

As introduced in Section 1.3.1, the available tools for measuring obesity (e.g., BMI, lean-to-fat mass ratio, waist-to-hip ratio, percentage body fat) are not always congruent in their conclusions, which can confound findings between different studies and patient outcomes in practice. For instance, in a large case-control study with participants from 52 countries, the waist-to-hip ratio has been found to be most predictive of myocardial infarction compared to other measures of obesity (e.g., BMI; Yusuf et al., 2005). In another study, a similar number of male and female participants has been reported with the BMI scale, but females are less likely to be classified as 'obese' by the waist-to-hip ratio (Gandhi et al., 2010). Cairu Li et al. (2006) also reported that, for women, waist-to-hip ratio, but not BMI, was predictive of cardiovascular disease incidence, whereas, in men, neither BMI nor waist-to-hip ratio was predictive of cardiovascular disease.

Further complicating matters, an obesogenic phenotype is not always predictive of negative metabolic health consequences (reviewed by Blüher, 2020), although the presence of obesity has been estimated to preclude changes in metabolic parameters in 30 to 50% of people (G. I. Smith et al., 2019). People classified as having a 'normal' BMI can still meet diagnostic criteria for metabolic syndrome (e.g., one study found metabolic syndrome in

17% of participants with a 'normal' BMI; Suliga et al., 2016). It has also been reported that a subset of people (10 – 30%) who are classified as 'obese' may not have concurrently presenting metabolic syndrome (i.e., three or more clinical markers) nor any metabolic dysfunction (Blüher, 2020). Although there can be a delay in the onset of metabolic syndrome in many people in this group (Blüher, 2020), some people with obesity do not develop cardiovascular and metabolic complications. For instance, after 20 years, Bell et al. (2015) reported that only about 50% of their sample with this 'metabolically healthy obesity' progressed to 'metabolically unhealthy obesity'. Overall, individual differences in the presentation of obesity can be further complicated by the limitations of these anthropomorphic tools. The idea that people can present with metabolic syndrome without concurrent obesity, or vice versa, is an important consideration as research continues.

4.5.4 Individual Differences in Anxiety

Like with human obesity, where individual differences in the associated consequences of excess weight exist, anxiety can vary depending on individual characteristics and life experience. Considering that the ability for a fearful, or even anxious, response to a threatening situation is evolutionarily considered to be beneficial in the short-term (i.e., when a threat is imminent or perceived to be imminent; Morris, 2019), it is relevant that in some people these responses become dysregulated (e.g., prolonged, activated in unwarranted situations) to the point of a clinical disorder (Meacham & Bergstrom, 2016). As theorized by Meacham and Bergstrom (2016), anxiety responses are, in part, a result of learning from exposure to environmental stimuli and would be highly dependent on an individual's specific environment. Individual differences in anxiety could also stem from some people being too sensitive to the environmental cues they encounter (Meacham & Bergstrom, 2016). There are also individual differences in the symptom profiles, type of anxiety disorder that is present (e.g., GAD, social anxiety disorder), and comorbid conditions occurring with the anxiety. For example, one study reported a 12-month prevalence of 18% for any anxiety disorder (i.e., DSM-IV criteria, including PTSD and obsessive-compulsive disorder), but severity of symptoms was increased in participants with a comorbid condition (e.g., major depressive disorder; Kessler et al., 2005).

Two important differences to consider when discussing the etiology, prevalence, and severity of anxiety disorders are gender and biological sex. Depending on the type of anxiety disorder being discussed, females are often reported to be at increased risk compared to males (reviewed by Christiansen, 2015). One explanation for this sex difference lies in sex hormone levels (e.g., estrogen, testosterone, progesterone). Indeed, testosterone, which is almost always higher in males, has been described as anxiolytic, whereas estrogen levels have been shown to interact with previous stress exposure to affect anxiety (Maeng & Milad, 2015). For instance, higher or more consistent estrogen levels might be protective with respect to anxiety development after traumatic experiences (Glover et al., 2012; Wharton et al., 2012). In contrast, progesterone seems to enhance arousal and anxiety responses (Maeng & Milad, 2015), and it has been reported that some people might be more sensitive to these mental health-related side effects of endogenous or exogenous progesterone (Reynolds et al., 2018; Standeven et al., 2020). Even though the exact mechanisms and risk factors for disordered anxiety continue to be elucidated, current research is highlighting the importance of considering individual differences in the development of, presentation of, and treatment options for anxiety disorders.

4.5.5 The Gut Microbiota and Personalized Medicine

As Bäckhed and colleagues (2005) describe, the gut microbiota can be considered a "microbial organ placed within a host organ" (p. 1915) due to its functionality in affecting energy balance and physiological processes in the host. Indeed, these microbes have been consistently demonstrated to work alongside the host in regulating the function of various key bodily systems (e.g., immune, reproductive, digestive, nervous; Martinez-Guryn et al., 2018; Qi et al., 2021; Sanidad et al., 2022) and in affecting health and disease development (Guo et al., 2012; Lynch & Pedersen, 2016; Moloney et al., 2014). One current question in microbiota research is on the stability of the gut microbiota, or how changeable any individual's microbiota would be and how this stability could affect host physiology (Ursell et al., 2012). Environmental factors such as diet and microbial exposure, along with genetic makeup and age of hosts, can broadly explain individual diversity in the gut microbiota, but overarching specific influences of gut microbiota composition are still

being explored (The Human Microbiome Project Consortium, 2012). As such, comprehensive mechanisms for why these microbes affect individual physiology and behaviour continue to be elucidated, but hormonal, neural, immune, and metabolic pathways are involved in the complex interplay between the gut, brain, gut microbiota, and associated host states (Suganya & Koo, 2020).

Above and beyond strain-specific effects of probiotics (see Section 4.2.1), there are individual host effects of probiotic treatment that are critical to study when trying to elucidate why probiotics seem to benefit specific health outcomes. The gut microbiota of land animals and humans is mainly comprised of Gram-positive bacteria, but the types of bacteria can vary greatly depending on the organism being examined (e.g., *Bifidobacterium* is highly present in human feces, *Lactobacilli* in pig feces; outlined in detail in Gatesoupe, 1999), which means the effects of probiotics could differ due to what is already present as part of the gut microbiota. Fish, molluscs, and arthropods have more Gram-negative bacteria in their gut microbiota, and as cautioned by Gatesoupe (1999), this fundamental compositional difference should be considered when designing and studying probiotic effects in land animals compared to water animals.

Rodent strain differences can also impact the function of probiotics that are studied. One study showed that probiotics were better able to colonize intestinal mucosa in male Swiss-Webster germ-free, compared to male C57BL/6JOlaHsd mice, while also demonstrating that there are extensive individual differences in probiotic impacts on humans depending on many factors (e.g., baseline gut microbiota; Zmora et al., 2018). In humans, the exact composition and function of the gut microbiota may also affect the response to probiotics or other drugs, which could affect treatment plans for specific individuals (Y. Chen et al., 2021). Various host characteristics have also been linked to the composition of the gut microbiota (e.g., country lived, alcohol consumption, diet, sex), which can complicate the interpretations of studies on probiotic effects and studies that attempt to link abundances of specific taxa with health or disease outcomes (Vujkovic-Cvijin et al., 2020). In fact, the ability to characterize and readily study changes in an individual's gut microbiota has been proposed to be of paramount importance for personalized medicine (e.g., customized therapies, side effect management; Saez-Lara et al., 2015; Ursell et al., 2012).

4.5.6 Protective Factors and Ways to Promote Health

Research in the field of epigenetics highlights the importance of early life exposures in affecting the body's response to different environmental risk and protective factors. However, studies also demonstrate that plasticity in the epigenome exists (i.e., epigenetic drift), that health is not exclusively static, and that there are protective factors (e.g., sleep, healthy diet, physical activity) for some individuals that can mitigate disease risk even in light of both genetic and environmental risk factors (Declerck & Vanden Berghe, 2018; Langley-Evans, 2015; McEwen, 2016). There are greater than 300 SNPs linked to obesity outcomes, but increasing physical activity and modifying diet can aid in reducing the risk of obesity even with SNP presence (Goodarzi, 2018). For instance, Herle et al. (2023) reported that increasing physical activity in children could help to prevent obesity in those that are at an increased genetic risk for obesity development. Furthermore, in people with a genetic risk for non-alcoholic fatty liver disease, improvements in diet can reduce liver fat and might be a preventative strategy for disease development in this group (J. Ma et al., 2018). Increasing the number of fruits and vegetables (e.g., berries, citrus, leafy green vegetables), more so in people with a higher genetic risk for obesity, can also result in decreased BMI (Z.-B. Wang et al., 2019). Relatedly, there is evidence that increasing sleep duration can also help to mitigate genetic risk for obesity (Watson et al., 2012).

Similar to the idea that there are protective factors for obesity development, the literature highlights that these interventions can help to mitigate the risk of mental health disorders. For instance, increased physical activity has been linked to reduced frequency of episodes of depression in individuals with increased genetic risk (K. W. Choi et al., 2020). As well, increased physical exercise has been reported to reduce the risk of PTSD in military veterans in response to the presence of one specific SNP (i.e., the Val66Met SNP in the BDNF gene that reduces BDNF protein and increases risk of anxiety- and stress-related disorders; B. L. Pitts et al., 2019). A recent meta-review concluded that physical activity is a major preventative strategy for all mental health conditions studied (e.g., depression, anxiety disorders), and there is evidence for other environmental risk factors affecting the development of mental health disorders when combined with genetic risk (i.e., tobacco smoking, poor diet, poor sleep; Firth et al., 2020).

A detailed account of how diet impacts the microbiota and associated health outcomes is provided in Chapter 1 of this dissertation (see Sections 1.1.5, 1.2.7, 1.3.5, and 1.4.6). As reviewed by Codella et al. (2018), exercise in both animal (e.g., swimming, voluntary wheel) and human studies (e.g., studying elite athletes, measuring cardiorespiratory fitness) improves various physiological and behavioural outcomes (e.g., increases butyrate, improves cognition, quicker muscle turnover) with direct links to the gut microbiota (e.g., increases *Lactobacillus*, *A. muciniphila*, diversity of microbiota taxa). As highlighted by the authors, further research is necessary to determine if different types of exercise (e.g., intensity, consistency) have distinct effects in certain groups of people (Codella et al., 2018). It would also be of benefit to increase research on the interactive effects of diet and exercise in different subject types (e.g., healthy participants, participants with metabolic syndrome or with other diseases or disorders; Codella et al., 2018). Sleep and the gut microbiota are also heavily bi-directionally interrelated, and it is well-accepted that sleep quality impacts health status (e.g., immune function, metabolic functioning, mental health; Han et al., 2022). For instance, presence of the genus *Faecalibacterium* in stool has been reported to be positively associated with sleep quality, health status, and well-being (Evans et al., 2017). Overall, lifestyle strategies to improve health, such as consuming a diet comprised of high-quality foods, increasing physical activity, and increasing sleep duration, can help to diminish the risk of disease development, in part, via epigenetic modifications that continue to be elucidated. Current research in this area highlights that these factors also interact with the gut microbiota to affect health status and that individual differences are a key consideration in elucidating these findings.

4.6 Implications and Concluding Remarks

This body of work demonstrates specific health-related alterations in response to nutritional factors (i.e., WD administration and CEREBIOME® treatment). Evidently, decisions made during experimental design (e.g., types of controls for the experimental manipulations, behavioural assays, strain or sex of rodent) can impact study reproducibility and comparability. These decisions warrant careful consideration because therapeutic manipulations that target the gut microbiota are vital for increasing our understanding of

the etiology, prevention, and treatment of conditions like obesity and anxiety. As outlined by Martinez et al. (2017), interactions between dietary components, individual host physiology, and the gut microbiota can promote or protect against obesity and metabolic disease. Similarly, it is well-accepted that the gut microbiota, directly and indirectly, impacts neurotransmitter (and precursor) levels, which affects brain function and the associated risk of disease and psychological disorders (e.g., autism spectrum disorder, Alzheimer's disease; Y. Chen et al., 2021). The characterization of these systems and proposed mechanisms can provide insight into why the gut microbiota and associated metabolites affect behavioural and molecular mechanisms in hosts, which can inform treatment and prevention strategies. For instance, if gut microbes could be manipulated by altering diet sources to increase host energy usage rather than energy storage, then this creates a potential treatment or prevention option for obesity and metabolic syndrome (reviewed by Cani et al., 2008; Dabke et al., 2019).

When it comes to studying determinants of health related to host-microbiota interactions, epigenetic, transcriptomic, and proteomic (e.g., post-translational modifications of proteins) approaches can all increase our understanding of the underlying processes that are affecting risk of disease (Al-Amrani et al., 2021; Lerner et al., 2016; Stiemsma & Michels, 2018). As stressed throughout this dissertation, human anxiety and obesity are prevalent and interrelated conditions that have been demonstrated to have overlapping physiological etiologies that depend on specific determinants of health (e.g., see de Wit et al., 2022; G. Zhao et al., 2009). Important to consider is that humans and animals harbour an exclusive collection of genes, microbiota patterns, and environmental exposures that interact to affect their individual health. As such, the study of specific determinants of health and disease and potential treatment or preventative options should be cognizant that people and animal model organisms are unique and will not always respond to manipulations designed to impact their health in predictable or similar ways. Moreover, in the life sciences, there has been, and continues to be, a strong preference for men and males in research. With the continued absence of females, women, and other gender identities in the literature (see Geller et al., 2018), our sex-specific findings in a Long–Evans rat model provide additional support for the idea that, together as researchers, we have a responsibility to ensure our research is more broadly applicable.

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APPENDIX A CHAPTER 2 SUPPLEMENTARY FILES

Table S2.1 Ingredient composition for the commercially available purified diets administered to rats in this study, including the grams of each ingredient relative to a total weight and the associated kilocalories (kcals), if applicable. **Note: Supplementary Table S1 in manuscript.**

Ingredient	**High-Carbohydrate Control Diet (D14042701)**		**Western Diet (D12079B)**	
	Grams	kcal	Grams	kcal
Casein, 80 Mesh	195	780	195	780
DL-Methionine	3	12	3	12
Corn Starch[a]	695	2780	50	200
Maltodextrin 10[a]	150	600	100	400
Sucrose[a]	0	0	341	1364
Cellulose, BW200	50	0	50	0
Milk Fat, Anhydrous[a]	42.5	383	200	1800
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[a]	0	0	1.5	0
Total	**1196.54**	**4685**	**1001.54**	**4686**

[a]These ingredients are present in different amounts between the two diets.

Table S2.2 Non-significant results of the sex by diet (2 × 3) factorial ANOVAs or ANCOVAs for all anxiety-related and defensive behaviours, including *F*-values, *p*-values, and effect sizes (η^2). **Note: Supplementary Table S2 in manuscript.**

Behavioural Variable	Result	*F*-value	*p*-value	η^2
1. Line Crosses[a]	Sex Main Effect	0.87	0.356	0.015
	Diet Main Effect	0.64	0.531	0.022
	Sex × Diet	1.00	0.373	0.035
2. Supported Rearing[a]	Sex Main Effect	0.92	0.341	0.016
	Diet Main Effect	0.85	0.432	0.030
	Sex × Diet	0.52	0.598	0.018
3. Time in Center (s)	Sex Main Effect	0.40	0.530	0.007
	Diet Main Effect	0.51	0.603	0.019
	Sex × Diet	0.05	0.951	0.002
4. Latency from Center (s)	Sex Main Effect	2.28	0.137	0.039
	Diet Main Effect	1.51	0.230	0.052
	Sex × Diet	0.22	0.800	0.008
5. Center Entries	Sex Main Effect	0.82	0.368	0.015
	Diet Main Effect	0.48	0.624	0.017
	Sex × Diet	0.36	0.697	0.013
6. Time in Hide Box (s)	Sex Main Effect	1.66	0.203	0.029
	Diet Main Effect	0.19	0.825	0.007
	Sex × Diet	1.27	0.289	0.045
7. Hide Box Entries	Sex Main Effect	2.43	0.125	0.041
	Diet Main Effect	0.73	0.485	0.025
	Sex × Diet	0.93	0.401	0.032
8. Thigmotaxis	Sex Main Effect	1.29	0.262	0.023
	Diet Main Effect	0.28	0.759	0.010
	Sex × Diet	0.95	0.393	0.034
9. Hide Box Entry Latency (s)	Sex Main Effect	1.88	0.176	0.032
	Diet Main Effect	0.33	0.722	0.011
	Sex × Diet	1.52	0.228	0.052
10. Latency to Re-Enter Center (s)	Sex Main Effect	0.10	0.758	0.002
	Diet Main Effect	0.62	0.544	0.023
	Sex × Diet	0.81	0.450	0.030
11. General Risk Assessment	Sex Main Effect	1.64	0.206	0.029
	Diet Main Effect	0.66	0.522	0.023
	Sex × Diet	0.08	0.919	0.003

[a]Since these behaviours are locomotion-related, ANCOVAs were conducted (covariate was weight at P56). s = seconds, "x" denotes the interaction of Sex and Diet.

Table S2.3 Descriptive statistics, split by sex, for all 14 behavioural variables in this study, including *n* after outlier removal, means, standard deviations (*SD*), minimum, and maximum. **Note: Supplementary Table S3 in manuscript.**

Behavioural Variable	Sex	*n*	Mean	*SD*	Min.	Max.
1. Line Crosses	F	30	82.77	31.28	13.00	158.00
	M	29	86.59	37.76	20.00	165.00
2. Supported Rearing	F	30	36.17	16.41	5.00	60.00
	M	29	35.55	15.78	9.00	76.00
3. Unsupported Rearing	F	30	2.00	2.12	0.00	6.00
	M	28	4.82	4.60	0.00	17.00
4. Time in Center (s)	F	30	33.63	16.02	1.98	79.48
	M	28	36.35	16.92	7.70	70.73
5. Latency from Center (s)	F	30	16.35	15.74	0.55	57.96
	M	28	11.22	9.30	0.76	35.23
6. Center Entries	F	30	9.53	4.54	1.00	20.00
	M	28	10.79	5.29	1.00	23.00
7. Time in Hide Box (s)	F	29	197.46	87.20	23.22	528.00
	M	29	158.55	131.19	0.00	425.30
8. Head Outs from Hide Box	F	30	7.80	3.88	1.00	18.00
	M	29	5.62	3.90	0.00	12.00
9. Hide Box Entries	F	30	4.73	2.32	2.00	11.00
	M	29	3.69	2.70	0.00	10.00
10. Thigmotaxis	F	29	7.09	6.14	0.00	23.25
	M	29	9.14	7.55	1.29	29.89
11. Hide Box Entry Latency (s)	F	30	105.77	106.33	7.00	416.00
	M	29	164.31	195.67	11.00	600.00
12. Latency to Re-Enter Center (s)	F	29	40.55	54.96	2.00	273.00
	M	28	35.68	37.87	5.00	201.00
13. Stretch Attend Postures	F	29	4.83	4.12	0.00	20.00
	M	29	7.86	3.83	1.00	16.00
14. General Risk Assessment	F	30	1.47	1.46	0.00	6.00
	M	29	2.00	1.63	0.00	6.00

s = seconds, F = female, M = male.

Table S2.4 Descriptive statistics, split by diet, for all 14 behavioural variables in this study, including *n* after outlier removal, means, standard deviations (*SD*), minimum, and maximum. **Note: Supplementary Table S4 in manuscript.**

	Variable	Diet	*n*	Mean	*SD*	Min.	Max.		Variable	Diet	*n*	Mean	*SD*	Min.	Max.
1.	Line Crosses	SD	19	86.37	30.92	24.00	165.00	8.	Head Outs from Hide Box	SD	19	6.90	3.38	1.00	13.00
		HCD	20	86.65	34.60	13.00	159.00			HCD	20	6.85	4.53	0.00	18.00
		WD	20	81.00	38.49	15.00	158.00			WD	20	6.45	4.19	0.00	13.00
2.	Supported Rearing	SD	19	36.74	17.05	5.00	76.00	9.	Hide Box Entries	SD	19	4.11	1.91	1.00	9.00
		HCD	20	37.80	13.49	11.00	59.00			HCD	20	4.75	3.08	0.00	11.00
		WD	20	33.10	17.56	9.00	64.00			WD	20	3.80	2.53	0.00	10.00
3.	Unsupported Rearing	SD	19	3.53	3.53	0.00	10.00	10.	Thigmotaxis	SD	19	8.04	7.50	0.00	25.76
		HCD	19	2.90	4.28	0.00	17.00			HCD	20	7.30	4.63	0.00	19.27
		WD	20	3.65	3.66	0.00	14.00			WD	19	9.04	8.38	1.48	29.89
4.	Time in Center (s)	SD	19	34.03	15.14	8.74	61.79	11.	Hide Box Entry Latency (s)	SD	19	111.05	107.90	7.00	416.00
		HCD	19	38.08	20.49	4.13	79.48			HCD	20	144.55	180.29	11.00	600.00
		WD	20	32.84	13.19	1.98	49.68			WD	20	146.85	178.86	23.00	600.00
5.	Latency from Center (s)	SD	19	14.38	12.78	0.73	47.03	12.	Latency to Re-Enter Center (s)	SD	19	48.37	34.94	5.00	132.00
		HCD	19	9.98	12.55	0.76	57.96			HCD	20	32.65	58.46	2.00	273.00
		WD	20	17.08	13.82	0.55	48.98			WD	18	33.50	44.55	6.00	201.00
6.	Center Entries	SD	18	9.39	3.68	3.00	17.00	13.	Stretch Attend Postures	SD	19	6.16	4.72	1.00	20.00
		HCD	20	11.00	5.32	2.00	23.00			HCD	20	6.50	3.86	0.00	16.00
		WD	20	9.95	5.53	1.00	20.00			WD	19	6.37	4.31	1.00	15.00
7.	Time in Hide Box (s)	SD	19	166.93	80.75	12.40	404.34	14.	General Risk Assessment	SD	19	1.47	1.43	0.00	5.00
		HCD	20	189.35	124.20	0.00	528.00			HCD	20	2.05	1.99	0.00	6.00
		WD	19	177.13	129.39	0.00	425.30			WD	20	1.65	1.14	0.00	4.00

s = seconds, SD = standard diet, HCD = high-carbohydrate diet, WD = Western diet.

Table S2.5 Pearson's correlations (n = 59) between significantly different behavioural variables (unsupported rearing, head outs, and stretch attend postures) in all rats (n = 57 – 58), along with subsets by sex and diet (n = 28 – 30, 19 – 20, 9 – 10). **Note: Supplementary Table S5 in manuscript.**

Subset	Unsupported Rearing & Head Outs (n)	Unsupported Rearing & Stretch Attend Postures (n)	Head Outs & Stretch Attend Postures (n)
All rats	-0.239 (58)	0.123 (57)	-0.434*** (58)
By sex			
M	-0.243 (28)	0.102 (28)	-0.535** (29)
F	-0.029 (30)	-0.259 (29)	-0.211 (29)
By diet			
SD	-0.060 (19)	-0.132 (19)	0.005 (19)
HCD	-0.103 (19)	0.039 (19)	-0.693*** (20)
WD	-0.515* (20)	0.485* (19)	-0.582** (19)
By sex and diet			
SD M	0.088 (9)	0.069 (9)	-0.551 (9)
SD F	0.208 (10)	-0.292 (10)	0.171 (10)
HCD M	0.062 (9)	-0.131 (9)	-0.515 (10)
HCD F	-0.105 (10)	-0.453 (10)	-0.735* (10)
WD M	-0.676* (10)	0.575 (10)	-0.618 (10)
WD F	-0.008 (10)	0.026 (9)	-0.487 (9)

*p < .05, **p < .01, ***p < .001; M = male, F = female, SD = standard diet, HCD = high-carbohydrate diet, WD = Western diet, BDNF = brain-derived neurotrophic factor. *Note.* Along with the rat that was removed from behavioural analyses for not moving during the 10-minute behavioural test (M SD), one outlier was removed each for unsupported rearing (HCD M) and stretch attend postures (WD F) before correlations were conducted.

Table S2.6 Pearson's correlations (n = 59) between unsupported rearing and key metabolic measures in all rats (n = 58), along with subsets by sex and diet (n = 28 – 30, 19 – 20, 9 – 10). **Note: Supplementary Table S6 in manuscript.**

Subset	Unsupported Rearing & Calorie Intake (*n*)	Unsupported Rearing & Weight Gain (*n*)	Unsupported Rearing & Leptin (*n*)	Unsupported Rearing & CA3 BDNF (*n*)
All rats	0.202 (58)	0.261* (58)	0.048 (58)	0.022 (58)
By sex				
M	-0.296 (28)	-0.281 (28)	-0.215 (28)	-0.057 (28)
F	0.257 (30)	0.147 (30)	0.255 (30)	-0.165 (30)
By diet				
SD	0.723*** (19)	0.575* (19)	0.538* (19)	-0.189 (19)
HCD	0.116 (19)	0.270 (19)	0.329 (19)	-0.006 (19)
WD	-0.086 (20)	0.062 (20)	-0.185 (20)	0.199 (20)
By sex and diet				
SD M	0.637 (9)	-0.194 (9)	0.420 (9)	-0.740* (9)
SD F	0.263 (10)	0.182 (10)	-0.360 (10)	-0.236 (10)
HCD M	-0.433 (9)	-0.076 (9)	0.069 (9)	0.123 (9)
HCD F	0.215 (10)	0.147 (10)	0.713* (10)	-0.431 (10)
WD M	-0.635* (10)	-0.475 (10)	-0.432 (10)	0.108 (10)
WD F	0.058 (10)	-0.170 (10)	-0.342 (10)	0.159 (10)

*p < .05, **p < .01, ***p < .001; M = male, F = female, SD = standard diet, HCD = high-carbohydrate diet, WD = Western diet, BDNF = brain-derived neurotrophic factor. *Note.* Along with the rat that was removed from behavioural analyses for not moving during the 10-minute behavioural test (M SD), one outlier was removed for unsupported rearing (HCD M).

Table S2.7 Pearson's correlations (n = 59) between head outs and key metabolic measures in all rats (n = 59), along with subsets by sex and diet (n = 29 – 30, 19 – 20, 9 – 10). **Note: Supplementary Table S7 in manuscript.**

Subset	Head Outs & Calorie Intake (n)	Head Outs & Weight Gain (n)	Head Outs & Leptin (n)	Head Outs & CA3 BDNF (n)
All rats	-0.068 (59)	-0.140 (59)	-0.076 (59)	0.160 (59)
By sex				
M	0.301 (29)	0.306 (29)	0.004 (29)	0.314 (29)
F	0.074 (30)	0.144 (30)	0.187 (30)	0.145 (30)
By diet				
SD	-0.173 (19)	-0.223 (19)	-0.259 (19)	-0.110 (19)
HCD	-0.189 (20)	-0.260 (20)	-0.395 (20)	0.340 (20)
WD	0.157 (20)	0.052 (20)	0.180 (20)	0.105 (20)
By sex and diet				
SD M	-0.376 (9)	-0.066 (9)	-0.295 (9)	0.033 (9)
SD F	0.277 (10)	0.006 (10)	-0.059 (10)	-0.075 (10)
HCD M	0.202 (10)	0.287 (10)	-0.560 (10)	0.537 (10)
HCD F	0.132 (10)	0.338 (10)	0.327 (10)	0.186 (10)
WD M	0.634* (10)	0.700* (10)	0.385 (10)	0.235 (10)
WD F	0.166 (10)	0.231 (10)	0.349 (10)	0.093 (10)

*p < .05, **p < .01, ***p < .001; M = male, F = female, SD = standard diet, HCD = high-carbohydrate diet, WD = Western diet, BDNF = brain-derived neurotrophic factor. *Note.* One rat was removed from behavioural analyses for not moving during the 10-minute behavioural test (M SD).

Table S2.8 Pearson's correlations (n = 59) between stretch attend postures and key metabolic measures in all rats (n = 58), along with subsets by sex and diet (n = 29, 19 – 20, 9 – 10). **Note: Supplementary Table S8 in manuscript.**

Subset	Stretch Attend Postures & Calorie Intake (*n*)	Stretch Attend Postures & Weight Gain (*n*)	Stretch Attend Postures & Leptin (*n*)	Stretch Attend Postures & CA3 BDNF (*n*)
All rats	0.246 (58)	0.313* (58)	0.242 (58)	-0.006 (58)
By sex				
M	-0.007 (29)	0.125 (29)	0.336 (29)	-0.043 (29)
F	-0.039 (29)	-0.255 (29)	-0.405* (29)	-0.213 (29)
By diet				
SD	0.142 (19)	-0.144 (19)	0.015 (19)	-0.317 (19)
HCD	0.440 (20)	0.611** (20)	0.513* (20)	-0.255 (20)
WD	0.227 (19)	0.472* (19)	0.273 (19)	0.424 (19)
By sex and diet				
SD M	0.263 (9)	-0.358 (9)	-0.328 (9)	-0.200 (9)
SD F	0.680* (10)	0.245 (10)	0.489 (10)	-0.529 (10)
HCD M	0.224 (10)	0.396 (10)	0.692* (10)	-0.577 (10)
HCD F	-0.337 (10)	-0.563 (10)	-0.723* (10)	0.014 (10)
WD M	-0.576 (10)	-0.606 (10)	-0.131 (10)	0.278 (10)
WD F	-0.520 (9)	-0.042 (9)	-0.369 (9)	-0.116 (9)

*p < .05, **p < .01, ***p < .001; M = male, F = female, SD = standard diet, HCD = high-carbohydrate diet, WD = Western diet, BDNF = brain-derived neurotrophic factor. *Note.* Along with the rat that was removed from behavioural analyses for not moving during the 10-minute behavioural test (M SD), one outlier was removed for stretch attend postures (WD F) before correlations were conducted.

Figure S2.1 Product sheet for the non-purified standard lab diet at the time it was used (2019 and 2021). **Note: Supplementary Figure F1 in manuscript.**

Laboratory Rodent Diet 5001*

DESCRIPTION

Laboratory Rodent Diet is recommended for rats, mice, hamsters and gerbils. This diet is formulated using the unique and innovative concept of Constant Nutrition®, paired with the selection of highest quality ingredients to assure minimal inherent biological variation in long-term studies. It is formulated for life-cycle nutrition; however, it is not designed for maximizing production in mouse breeding colonies. This product has been the standard of biomedical research for over 65 years.

Features and Benefits

- Constant Nutrition® formula helps minimize nutritional variables
- High quality animal protein added to create a superior balance of amino acids for optimum performance
- Formulated for multiple species for single product inventory
- The rodent diet standard for biomedical research

Product Forms Available

- Oval pellet, 10 mm x 16 mm x 25 mm length (3/8"x5/8"x1")
- Meal (ground pellets)

Other Versions Available

- 5010 Laboratory Auoclavable Rodent Diet
- 5L0D PicoLab Laboratory Rodent Diet (Minimum order required)

GUARANTEED ANALYSIS

Crude protein not less than	23.0%
Crude fat not less than	4.5%
Crude fiber not more than	6.0%
Ash not more than	8.0%

INGREDIENTS

Ground corn, dehulled soybean meal, dried beet pulp, fish meal, ground oats, brewers dried yeast, cane molasses, dehydrated alfalfa meal, dried whey, wheat germ, porcine animal fat preserved with BHA, porcine meat meal, wheat middlings, salt, calcium carbonate, DL-methionine, choline chloride, cholecalciferol, vitamin A acetate, folic acid, menadione dimethylpyrimidinol bisulfite (source of vitamin K), pyridoxine hydrochloride, biotin, thiamin mononitrate, nicotinic acid, calcium pantothenate, dl-alpha tocopheryl acetate, vitamin B_{12} supplement, riboflavin, ferrous sulfate, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, sodium selenite.

FEEDING DIRECTIONS

Feed ad libitum to rodents. Plenty of fresh, clean water should be available to the animals at all times.

Rats- All rats will eat varying amounts of feed depending on their genetic origin. Larger strains will eat up to 30 grams per day. Smaller strains will eat up to 15 grams per day. Feeders in rat cages should be designed to hold two to three days supply of feed at one time.

Mice-Adult mice will eat up to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal. Feed should be available on a free choice basis in wire feeders above the floor of the cage.

Hamsters-Adults will eat up to 14 grams per day.

04/05/12

CHEMICAL COMPOSITION[1]

Nutrients[2]	
Protein, %	**23.9**
Arginine, %	1.41
Cystine, %	0.31
Glycine, %	1.21
Histidine, %	0.57
Isoleucine, %	1.14
Leucine, %	1.83
Lysine, %	1.41
Methionine, %	0.67
Phenylalanine, %	1.04
Tyrosine, %	0.71
Threonine, %	0.91
Tryptophan, %	0.29
Valine, %	1.17
Serine, %	1.19
Aspartic Acid, %	2.81
Glutamic Acid, %	4.37
Alanine, %	1.43
Proline, %	1.49
Taurine, %	0.02
Fat (ether extract), %	**5.0**
Fat (acid hydrolysis), %	**5.7**
Cholesterol, ppm	200
Linoleic Acid, %	1.22
Linolenic Acid, %	0.10
Arachidonic Acid, %	<0.01
Omega-3 Fatty Acids, %	0.19
Total Saturated Fatty Acids, %	1.56
Total Monounsaturated Fatty Acids, %	1.60
Fiber (Crude), %	**5.1**
Neutral Detergent Fiber[3], %	15.6
Acid Detergent Fiber[4], %	6.7
Nitrogen-Free Extract (by difference), %	**48.7**
Starch, %	31.9
Glucose, %	0.22
Fructose, %	0.30
Sucrose, %	3.70
Lactose, %	2.01
Total Digestible Nutrients,%	**76.0**
Gross Energy, kcal/gm	**4.07**
Physiological Fuel Value[5], kcal/gm	**3.36**
Metabolizable Energy, kcal/gm	**3.02**
Minerals	
Ash, %	**7.0**
Calcium, %	0.95
Phosphorus, %	0.66
Phosphorus (non-phytate), %	0.39
Potassium, %	1.18
Magnesium, %	0.21
Sulfur, %	0.36
Sodium, %	0.40
Chlorine, %	0.67
Fluorine, ppm	16
Iron, ppm	270
Zinc, ppm	79
Manganese, ppm	70
Copper, ppm	13
Cobalt, ppm	0.90
Iodine, ppm	1.0
Chromium, ppm	1.2
Selenium, ppm	0.30
Vitamins	
Carotene, ppm	2.3
Vitamin K (as menadione),ppm	1.3
Thiamin Hydrochloride, ppm	16
Riboflavin, ppm	4.5
Niacin, ppm	120
Pantothenic Acid, ppm	24
Choline Chloride, ppm	2250
Folic Acid, ppm	7.1
Pyridoxine, ppm	6.0
Biotin, ppm	0.30
B_{12}, mcg/kg	50
Vitamin A, IU/gm	15
Vitamin D_3 (added), IU/gm	4.5
Vitamin E, IU/kg	42
Ascorbic Acid, mg/gm	—
Calories provided by:	
Protein, %	28.507
Fat (ether extract), %	13.496
Carbohydrates, %	57.996

*Product Code

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
3. NDF = approximately cellulose, hemi-cellulose and lignin.
4. ADF = approximately cellulose and lignin.
5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate (use Nitrogen Free Extract) x 4,9,4 kcal/gm respectively.

LabDiet
www.labdiet.com

APPENDIX B CHAPTER 3 SUPPLEMENTARY FILES

Table S3.1 PCR efficiencies (%) for all primers (i.e., RPL13A reference gene, GAPDH reference gene, GR target gene, and NPY target gene).

Plate (ID)	RPL13A %Efficiency	GAPDH %Efficiency	GR %Efficiency	NPY %Efficiency
1 (Adrenals_GR1)	110.8	111.4	107.6	N/A
2 (Adrenals_GR2)	111.0	112.1	111.0	N/A
3 (Adrenals_NPY1)	116.3	115.0	N/A	117.3
4 (Adrenals_NPY2)	109.0	113.2	N/A	111.7
5 (Hippocampus_GR1)*	113.8	109.6	116.0	N/A
6 (Hippocampus_GR2)*	114.9	111.7	116.6	N/A
7 (Hippocampus_NPY1)*	117.0	115.4	N/A	113.2
8 (Hippocampus_NPY2)*	114.7	114.2	N/A	111.1

*Due to capacity limitations on plates 1 – 4, 8 of 80 adrenal cDNA samples were added to plates 5 – 8, which were concurrently run RT-qPCR plates (same primers and protocol) as part of another project using hippocampal CDNA. *Note*. PCR Efficiencies are calculated for each primer from three duplicate points (i.e., the 1 in 5, 1 in 50, and 1 in 500 pooled cDNA dilutions). RPL13A = ribosomal protein L13a, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, GR = glucocorticoid receptor, NPY = neuropeptide Y.

Table S3.2 Pearson's correlations between fasting weight loss and metabolic hormone (ghrelin, leptin, and glucagon) levels, GR gene expression, and NPY gene expression (n = 78 – 79), including all subsets by treatment, diet, and/or sex (n = 9 – 40).

Subset (*n*)	**Fasting Weight Loss & Ghrelin**	**Fasting Weight Loss & Leptin**	**Fasting Weight Loss & Glucagon**	**Fasting Weight Loss & GR Expression**	**Fasting Weight Loss & NPY Expression**
All rats (78–79)	-0.116	-0.287*	-0.234*	-0.303**	-0.111
By treatment					
PL (39–40)	-0.171	-0.275	-0.116	-0.466**	-0.212
PR (38–40)	-0.063	-0.305	-0.339*	-0.076	-0.022
By diet					
SD (39–40)	-0.306	0.296	-0.229	-0.252	-0.040
WD (38–40)	-0.090	0.346*	-0.189	-0.451**	-0.311
By sex					
M (39–40)	-0.120	-0.598***	-0.092	-0.066	0.083
F (39–40)	0.281	-0.390*	-0.267	-0.274	-0.221
By treatment, diet, and sex					
PL SD M (10)	0.286	0.510	-0.046	-0.591	-0.548
PL SD F (9–10)	-0.347	-0.478	-0.392	-0.264	0.217
PL WD M (9–10)	-0.130	0.042	0.329	0.434	-0.207
PL WD F (10)	-0.282	-0.522	-0.148	-0.282	-0.286
PR SD M (10)	-0.293	0.547	-0.174	-0.081	0.654*
PR SD F (10)	0.563	0.464	-0.232	0.033	-0.260
PR WD M (9–10)	0.264	0.039	-0.432	-0.339	0.036
PR WD F (9–10)	-0.219	0.311	-0.306	-0.123	-0.236

*p < .05, **p < .01, ***p < .001; PL = placebo, PR = probiotic, SD = standard diet, WD = Western diet, M = male, F = female, GR = glucocorticoid receptor, NPY = neuropeptide Y.

Table S3.3 Summary of significant correlations between calorie consumption during behavioural testing (NSFT and home cage observation) and specific metabolic measures (i.e., early adult weight, relative calorie intake, fasting weight loss; $n = 76 – 78$), including all subsets by treatment, diet, and/or sex ($n = 9 – 40$).

Subset (*n*)	**Adult Weight & Calories NSFT**	**Adult Weight & Calories Home Cage**	**Relative Calorie Intake & Calories Home Cage**	**Fasting Weight Loss & Calories NSFT**	**Fasting Weight Loss & Calories Home Cage**
All rats (76–78)	0.177	0.376***	0.374***	-0.176	0.149
By treatment					
PL (38–39)	0.045	0.423**	0.359*	-0.128	0.281
PR (37–40)	0.328*	0.334*	0.393*	-0.217	-0.003
By diet					
SD (40)	0.346*	0.288	0.260	0.237	0.346*
WD (36–38)	-0.030	0.425**	0.446**	-0.126	0.374*
By sex					
M (36–39)	0.116	0.028	0.059	-0.193	-0.269
F (39–40)	0.206	-0.303	-0.287	-0.454**	0.198
By treatment, diet, and sex					
PL SD M (10)	0.304	0.164	0.053	0.413	0.658*
PL SD F (10)	-0.578	-0.237	-0.087	-0.219	0.225
PL WD M (8–10)	-0.285	-0.575	-0.582	-0.353	-0.364
PL WD F (9–10)	-0.239	0.302	-0.034	-0.427	0.362
PR SD M (10)	-0.385	0.520	0.574	-0.670*	0.207
PR SD F (10)	-0.035	-0.609	-0.624	-0.027	-0.190
PR WD M (8–10)	0.513	-0.174	0.139	0.367	-0.088
PR WD F (10)	0.025	0.094	0.213	-0.060	-0.705*

*$p < .05$, **$p < .01$, ***$p < .001$; PL = placebo, PR = probiotic, SD = standard diet, WD = Western diet, M = male, F = female, NSFT = novelty-suppressed feeding task. *Note*. Calories consumed in the NSFT were not correlated with either relative calorie intake or calories in the home cage, overall or in any subgroups.

Table S3.4 Pearson's correlations between metabolic hormones (i.e., ghrelin, leptin, and glucagon) and GR and NPY gene expression (n = 77 – 79), including all subsets by treatment, diet, and/or sex (n = 8 – 40).

Subset (*n*)	**Ghrelin & Leptin**	**Ghrelin & Glucagon**	**Leptin & Glucagon**	**Ghrelin & GR Exp.**	**Ghrelin & NPY Exp.**	**Leptin & GR Exp.**	**Leptin & NPY Exp.**	**Glucagon & GR Exp.**	**Glucagon & NPY Exp.**	**GR Exp. & NPY Exp.**
All rats (77–79)	-0.010	0.056	0.211	0.007	-0.232*	-0.090	-0.127	0.226*	-0.038	0.278*
By treatment										
PL (38–39)	-0.065	0.008	0.232	0.024	-0.287	-0.090	-0.092	0.199	-0.248	0.189
PR (38–40)	0.062	0.094	0.197	-0.012	-0.173	-0.091	-0.175	0.277	0.118	0.411**
By diet										
SD (39–40)	0.094	0.024	0.238	-0.016	-0.343*	0.121	0.058	-0.033	-0.034	0.360*
WD (37–39)	0.152	0.118	0.146	0.057	-0.049	-0.343*	-0.325*	0.391*	-0.053	0.181
By sex										
M (38–39)	0.221	0.087	0.256	0.066	-0.206	-0.034	-0.165	0.012	-0.123	0.412**
F (38–40)	-0.064	-0.069	0.293	-0.247	-0.339*	0.008	-0.037	0.342*	0.004	0.152
By treatment, diet, and sex										
PL SD M (10)	0.144	0.076	0.117	0.165	-0.521	-0.220	0.120	-0.445	-0.109	0.429
PL SD F (9–10)	0.224	-0.329	0.340	-0.442	-0.432	0.457	-0.470	0.754*	0.056	0.056
PL WD M (8–10)	0.566	0.167	0.175	0.204	-0.318	0.116	-0.004	0.477	-0.689	0.050
PL WD F (10)	0.162	0.594	0.320	0.072	0.075	-0.309	-0.249	0.311	-0.398	-0.169
PR SD M (10)	-0.346	0.165	0.330	-0.224	-0.449	0.408	0.488	-0.121	-0.337	0.517
PR SD F (10)	0.611	-0.006	0.224	0.141	-0.314	0.051	-0.233	-0.106	0.100	0.595
PR WD M (9–10)	-0.043	-0.006	0.426	0.225	0.066	-0.046	-0.269	0.548	0.501	0.358
PR WD F (9–10)	0.468	-0.004	0.081	-0.341	-0.715*	-0.469	-0.429	0.487	0.132	0.317

*p < .05, **p < .01, ***p < .001; PL = placebo, PR = probiotic, SD = standard diet, WD = Western diet, M = male, F = female, Exp. = Expression, GR = glucocorticoid receptor, NPY = neuropeptide Y.

Table S3.5 Pearson's correlations between anxiety-related behavioural variables in the NSFT (n = 78 – 79), including all subsets by treatment, diet, and/or sex (n = 9 – 40).

Subset (*n*)	**Line Crosses & Supported Rears**	**Line Crosses & Center Entries**	**Line Crosses & Time in Center**	**Line Crosses & Latency to Center**	**Supported Rears & Center Entries**	**Supported Rears & Time in Center**	**Supported Rears & Latency to Center**	**Center Entries & Time in Center**	**Center Entries & Latency to Center**	**Time in Center & Latency to Center**
All rats (78–79)	0.638***	0.679***	-0.109	-0.194	0.275*	-0.357**	-0.157	0.334**	-0.292**	-0.131
By treatment										
PL (38–39)	0.737***	0.751***	0.092	-0.321*	0.472**	-0.123	-0.282	0.367*	-0.362*	-0.114
PR (40)	0.542***	0.628***	-0.279	-0.037	0.118	-0.542***	-0.034	0.274	-0.231	-0.176
By diet										
SD (39–40)	0.603***	0.533***	-0.261	-0.134	0.027	-0.409**	0.080	0.375*	-0.309	-0.254
WD (38–39)	0.641***	0.831***	0.183	-0.212	0.545***	-0.199	-0.372*	0.313	-0.288	-0.010
By sex										
M (39–40)	0.691***	0.613***	-0.023	-0.275	0.275	-0.282	-0.115	0.438**	-0.308	-0.260
F (38–39)	0.331*	0.680***	-0.052	0.058	0.052	-0.374*	-0.112	0.367*	-0.220	0.001
By treatment, diet, and sex										
PL SD M (10)	0.707*	0.625	-0.120	0.058	0.735*	-0.014	0.155	0.034	-0.138	-0.349
PL SD F (10)	0.665*	0.499	-0.133	-0.059	-0.114	-0.220	0.008	0.541	-0.308	-0.107
PL WD M (9–10)	0.742*	0.850**	0.415	-0.546	0.627	-0.106	-0.520	0.586	-0.536	-0.174
PL WD F (9)	0.609	0.870**	0.786*	0.506	0.748*	0.231	-0.051	0.567	0.271	0.578
PR SD M (10)	0.450	0.335	-0.685*	-0.139	-0.075	-0.693*	0.238	0.025	-0.259	-0.200
PR SD F (10)	-0.145	0.810**	0.137	-0.026	-0.525	-0.692*	0.309	0.545	-0.447	-0.610
PR WD M (10)	0.744*	0.731*	0.340	-0.187	0.371	0.168	-0.236	0.772**	-0.224	-0.531
PR WD F (10)	0.045	0.725*	-0.292	0.173	0.243	-0.631	-0.423	-0.168	-0.192	0.188

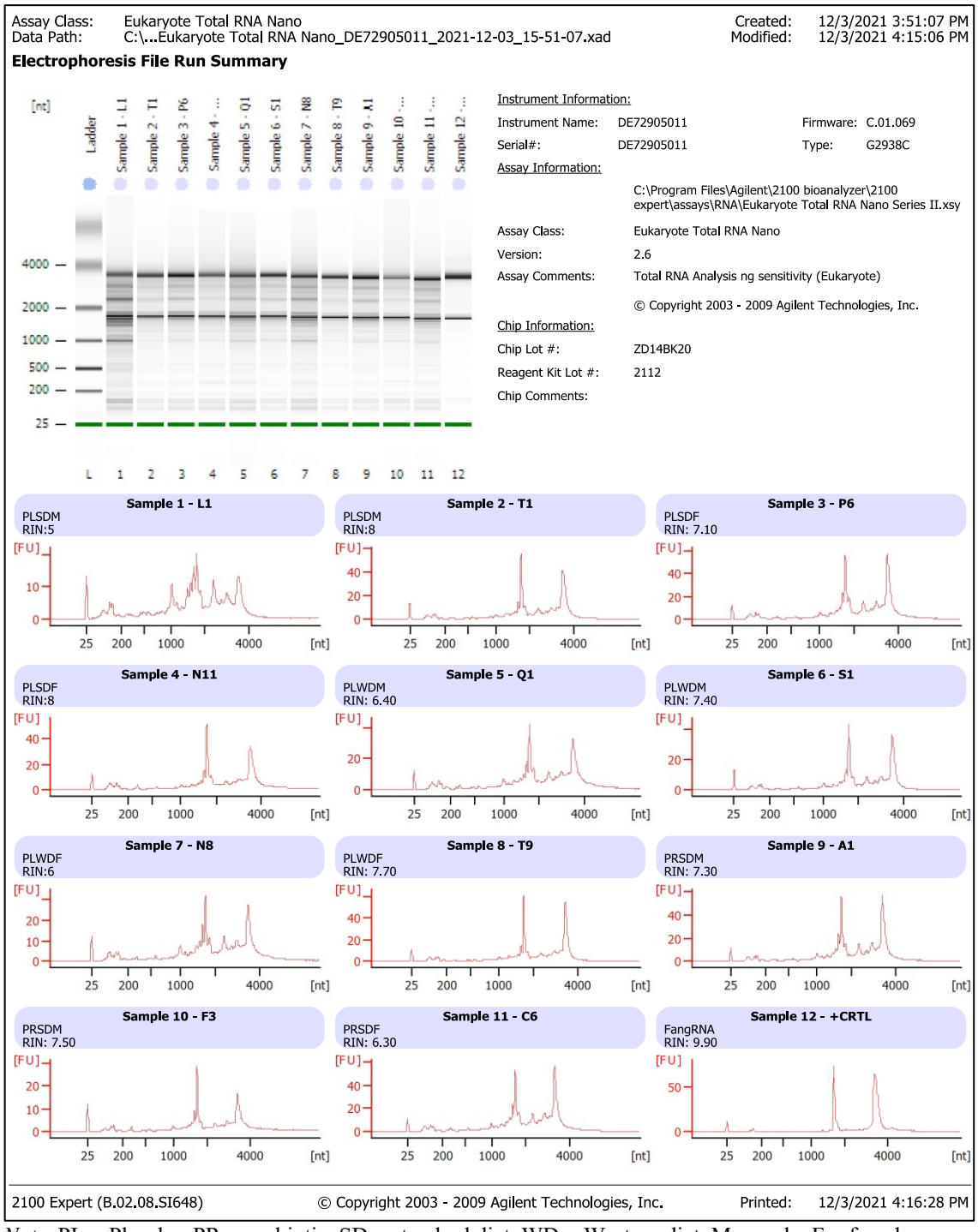
*p < .05, **p < .01, ***p < .001; PL = placebo, PR = probiotic, SD = standard diet, WD = Western diet, M = male, F = female, NSFT = novelty-suppressed feeding task**.** *Note.* No significant correlations overall between unsupported rearing and other behavioural variables were found, but unsupported rears and center entries were positively correlated (r = 0.683, p = .030, n = 10) in PR SD females. As well, unsupported rears and time in center were positively correlated (r = 0.634, p = .049, n = 10) in PR WD females.

Table S3.6 Pearson's correlations (n = 77 – 80) between adult weight, relative calorie intake, and fasting weight loss, and time taken to first eat (NSFT or home cage observation), along with calories consumed (NSFT or home cage) and time taken to eat (NSFT or home cage), including all subsets by treatment, diet, and/or sex (n = 8 – 40).

Subset (*n*)	**Adult Weight & Time to Eat NSFT**	**Adult Weight & Time to Eat Home Cage**	**Relative Calorie Intake & Time to Eat NSFT**	**Relative Calorie Intake & Time to Eat Home Cage**	**Fasting Weight Loss & Time to Eat NSFT**	**Fasting Weight Loss & Time to Eat Home Cage**	**Calories NSFT & Time to Eat NSFT**	**Calories Home Cage & Time to Eat Home Cage**
All rats (77–80)	0.044	-0.378***	0.056	-0.369***	0.047	-0.047	-0.572***	-0.504***
By treatment								
PL (38–40)	0.128	-0.407**	0.104	-0.434**	0.122	-0.016	-0.725***	-0.560***
PR (38–40)	-0.086	-0.346*	-0.054	-0.280	-0.071	-0.077	-0.370*	-0.443**
By diet								
SD (40)	-0.062	-0.178	-0.041	-0.219	-0.043	-0.226	-0.584***	-0.667***
WD (37–40)	0.143	-0.485**	0.150	-0.441**	0.216	-0.340*	-0.652***	-0.464**
By sex								
M (38–40)	-0.030	-0.307	-0.028	-0.266	-0.030	0.331*	-0.694***	-0.459**
F (39–40)	-0.196	0.016	-0.110	-0.102	0.034	0.001	-0.452**	-0.490**
By treatment, diet, and sex								
PL SD M (10)	0.175	-0.159	0.225	-0.098	-0.018	-0.229	-0.719*	-0.703*
PL SD F (10)	0.581	-0.161	0.607	-0.339	0.343	-0.042	-0.805**	-0.772**
PL WD M (8–10)	-0.097	0.435	-0.376	0.505	0.180	-0.017	-0.864**	-0.626
PL WD F (9-10)	-0.335	-0.036	-0.066	-0.164	-0.281	-0.235	-0.537	-0.080
PR SD M (10)	0.037	-0.323	0.113	-0.451	0.519	-0.312	-0.544	-0.723*
PR SD F (10)	-0.508	0.370	-0.466	0.348	-0.380	-0.175	-0.613	-0.725*
PR WD M (8-10)	-0.434	-0.139	-0.502	0.500	-0.069	0.112	-0.718*	-0.044
PR WD F (10)	0.189	-0.168	0.060	-0.171	0.564	0.458	-0.249	-0.624

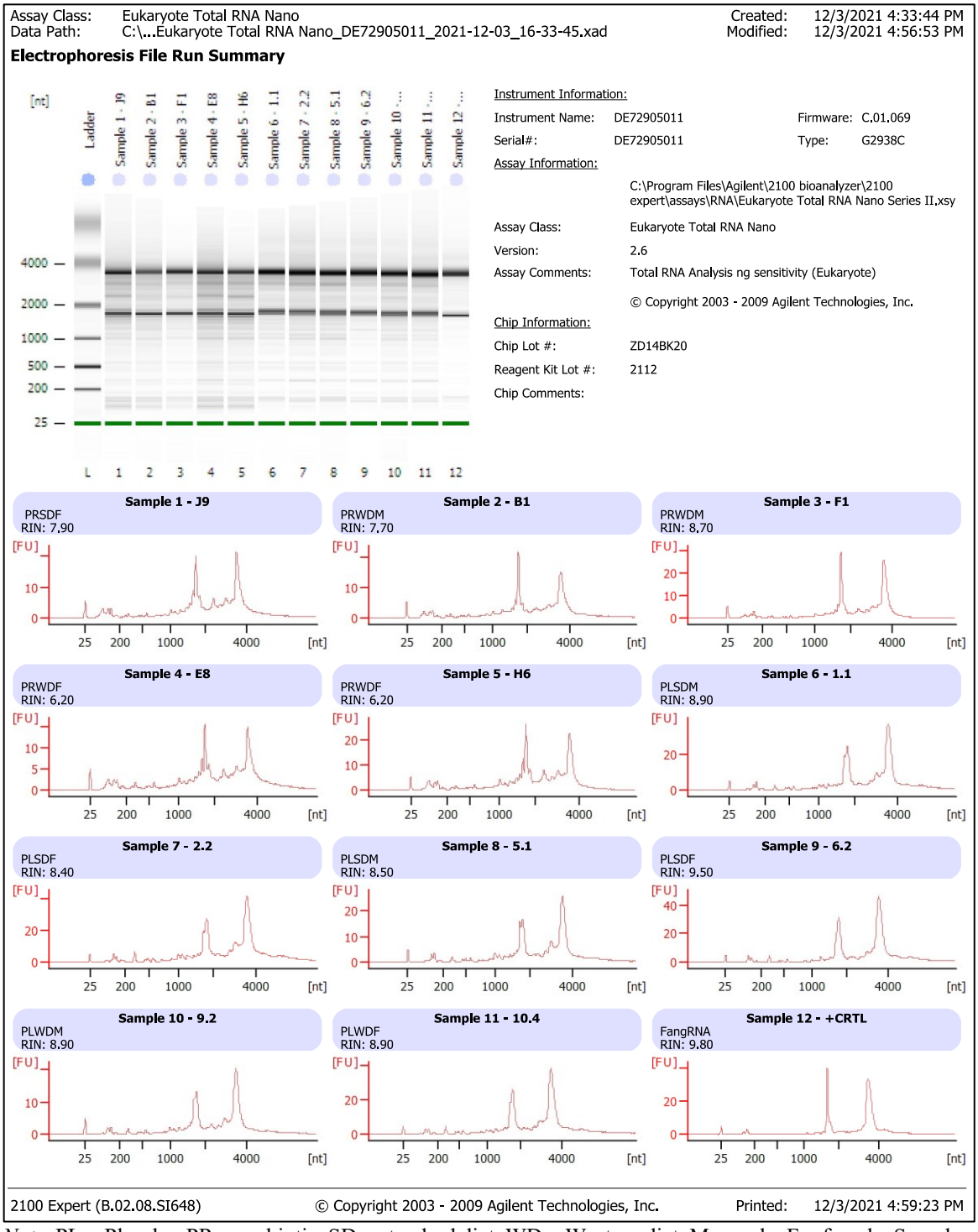
*p < .05, **p < .01, ***p < .001; PL = placebo, PR = probiotic, SD = standard diet, WD = Western diet, M = male, F = female, NSFT = novelty-suppressed feeding task**.** *Note.* No significant correlations were found overall or in any subsets for calories consumed in the NSFT and time to eat in the home cage, for calories consumed in the home cage and time to eat in the NSFT, and for time taken to eat in the NSFT and time taken to eat in the home cage.

Figure S3.1 Agilent bioanalyzer gel electrophoresis (eukaryotic total RNA) results for the first 11 adrenal RNA samples showing sample ID (i.e., L1, T1, P6, N11, Q1, S1, N8, T9, A1, F3, C6), experimental group (i.e., PL SD M, PL SD F, PL WD M, PL WD F, PR SD M, PR SD F), RNA integrity numbers (RINs; range = 5.00 – 8.00), including positive control RNA and nucleotide ladder for reference.



Note. PL = Placebo, PR = probiotic, SD = standard diet, WD = Western diet, M = male, F = female.

Figure S3.2 Agilent bioanalyzer gel electrophoresis (eukaryotic total RNA) results for the remaining 5 adrenal RNA samples showing sample ID (i.e., J9, B1, F1, E8, H6), experimental group (i.e., PR SD F, PR WD M, PR WD F), RNA integrity numbers (RINs; range = 6.20 – 8.70), including positive control RNA and nucleotide ladder for reference.



Note. PL = Placebo, PR = probiotic, SD = standard diet, WD = Western diet, M = male, F = female. Samples 6 through 11 were added to the chip but are part of another project.

Figure S3.3 Gel electrophoresis results of four neuropeptide Y (NPY) products (i.e., after RT-qPCR analysis) amplified by PCR to confirm one DNA product is present and of the expected size (i.e., 288 base pairs). A 2log ladder beginning at 100 nucleotides (0.1 kilobases) and a PCR water negative control are included. PCR was conducted with taq polymerase, with an initial denaturation of 94 °C (5 min). Denaturation was followed by 35 cycles of heating at 94 °C (30 s), annealing at 60 °C (30 s), and amplifying at 72 °C (1 min). PCR concluded with a 7-minute final extension at 72 °C. Products were kept at 4 °C until loaded onto the gel. A 1.0% agarose gel was prepared, with 5 µL of each sample loaded, and run at 100 V for 1 hour.

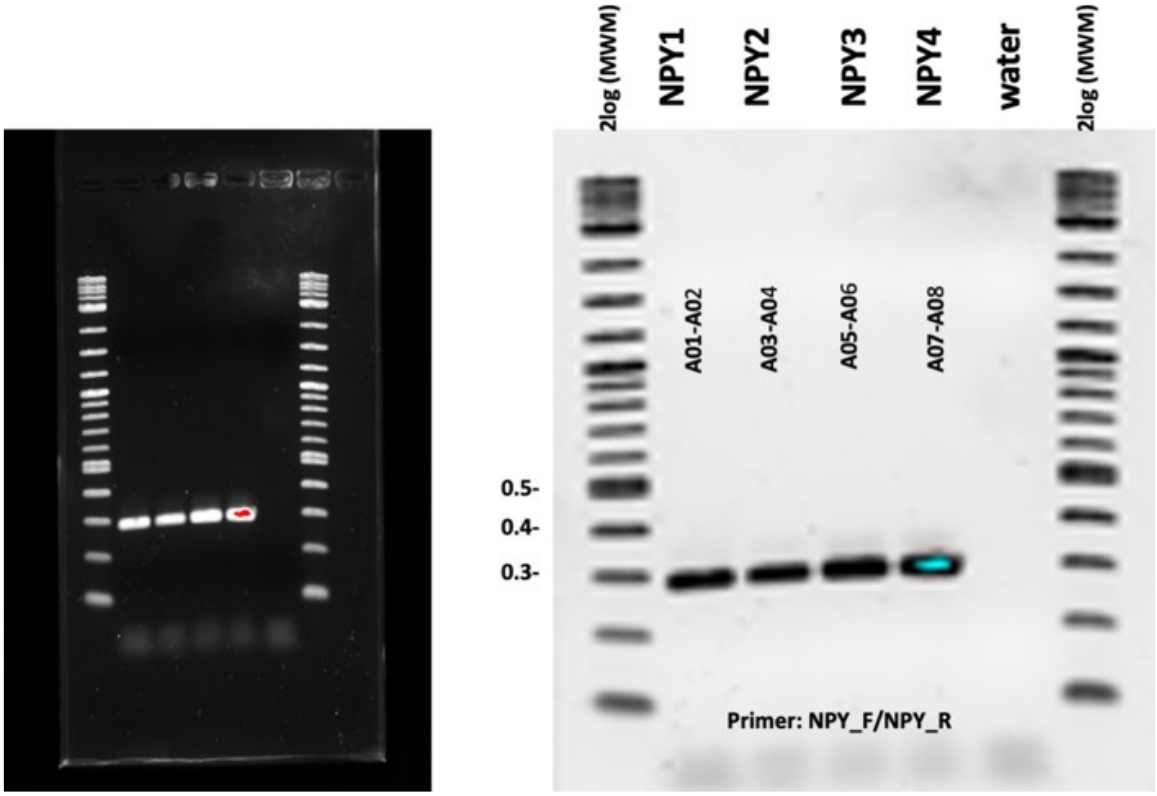
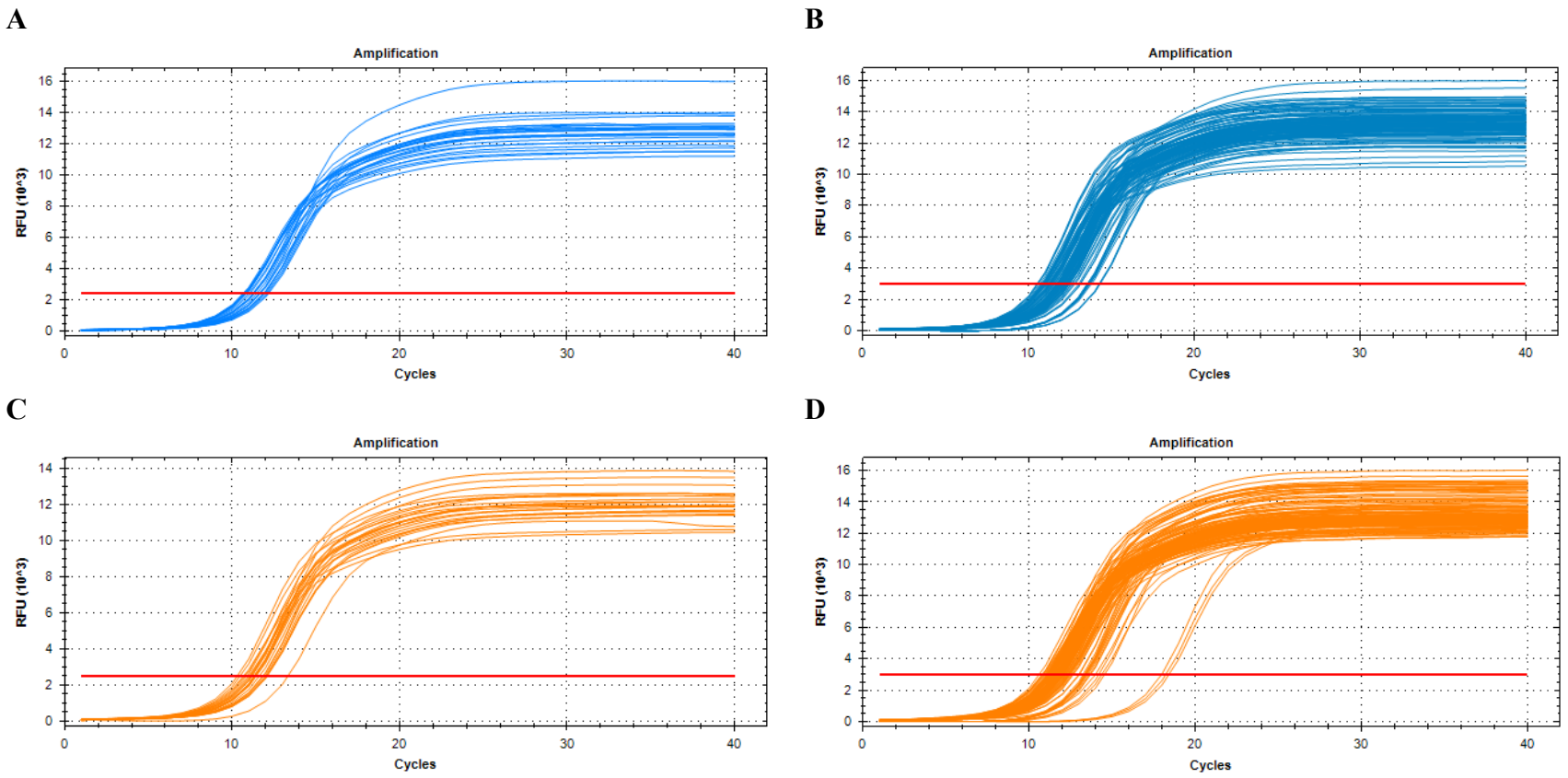


Figure S3.4 16S rRNA gene amplification plots from detection qPCR for **A.** Placebo mother rats (n = 8, in triplicate); **B.** Placebo offspring rats (n = 40, in triplicate); **C.** Probiotic mother rats (n = 8, in triplicate); and **D.** Probiotic offspring rats (n = 40, in triplicate), showing amplification of the bacterial 16S rRNA gene in all mother and offspring rats (indicating presence of bacterial DNA extracted from caecum samples). No amplification of the no template controls was found, and positive controls amplified as expected. *Note.* All three later Cqs in panel D are from the same probiotic rat (rat "E9").



APPENDIX C GENERALIZED LINEAR MODELS

For both Study 1 (Chapter 2) and Study 2 (Chapter 3), all dependent variables were examined for homogeneity of variance with Levene's test while conducting ANOVA or ANCOVA analyses as part of the jamovi statistical software package (Version 2.3.0; Fox & Weisberg, 2020; Lenth, 2020; R Core Team, 2021; The jamovi project, 2022). If Levene's test was found to be significant, ANOVA or ANCOVA findings were confirmed with generalized linear models in SPSS (GENLIN; version 28.0.1.1, IBM Statistics). For continuous data, all baseline (intercepts-only) models were tested with both normal distribution and link identity or gamma distribution and log identity to determine the more parsimonious (i.e., lowest Bayesian Information Criterion, BIC) distribution and link function combination. In cases where at least one zero was part of the continuous data (i.e., calories consumed in the NSFT), a normal distribution and link identity were automatically chosen. For count data (i.e., unsupported rears), baseline models with Poisson distribution and log identity or negative binomial with log identity were compared.

Then, the chosen distribution and link function combination with the lowest BIC was used to run generalized linear models with the independent variables (main effects) and their interactions included as specified model effects in a factorial non-nested design. For parameter estimation, Fisher scoring was selected as the method, maximum-likelihood was selected as the scale parameter method, and model-based estimator as the covariance matrix, with 100 max iterations, max step halving of 5, and parameter convergence and singularity tolerance selected. Wald (Type III) was selected as the analysis type, with the Wald confidence interval level at 95 and full likelihood for the log-likelihood function. *Post hoc* pairwise comparisons were analyzed with Sidak's test to adjust for multiple comparisons and missing data was excluded (as would have been done through outlier removal in the ANOVAs or ANCOVAs).

The following ANOVAs or ANCOVAs for Study 1 (Chapter 2) did not have significant *p* values for Levene's test: line crosses, supported rears, time in center, latency from center, center entries, head outs from hide box, hide box entries, thigmotaxis, center re-entry

latency, stretch attend postures, general risk assessment, food intake, absolute calorie intake, relative calorie intake, wean weight, and body weight. The following ANOVAs or ANCOVAs for Study 2 (Chapter 3) did not have significant *p* values for Levene's test: line crosses, supported rears, unsupported rears, latency to enter center, time in center, center entries, pellet contacts (perimeter), pellet contacts (center), pellet carries, time to eat (NSFT), feeding bouts (NSFT), food eaten (NSFT), time to eat (home cage), feeding bouts (home cage), wean weight, adult weight, relative calorie intake, calorie intake by cage, plasma glucagon, glucocorticoid receptor expression, and neuropeptide Y expression.

For Study 1, the variables unsupported rears, time in hide box, hide box entry latency, weight gain, plasma leptin, and CA3 BDNF had significant Levene's tests (i.e., unequal variances between groups). These variables were analyzed in a factorial generalized linear model as described above to confirm ANOVA findings. Model summaries are included in the below table (Table AC.1). For unsupported rearing, a main effect of sex (i.e., males performed more unsupported rears than females) was still revealed ($\chi2(1) = 8.77$, $p = .003$). For time in hide box and hide box entry latency, no significant main effects or interaction between diet and sex were revealed, as was also concluded by ANOVA analysis. For the variable weight gain, main effects of sex (i.e., males gained more weight than females; $\chi2(1) = 402.96$, $p < .001$) and diet (i.e., HCD and WD animals gained more weight than SD animals; $\chi2(1) = 40.98$, $p < .001$; Sidak significances for pairwise comparisons both $p < .001$), with no interaction between diet and sex, were still revealed. For plasma leptin, main effects of sex (i.e., males had higher leptin than females; $\chi2(1) = 46.36$, $p < .001$) and diet (i.e., HCD and WD animals had higher leptin than SD animals; $\chi2(1) = 134.68$, $p < .001$; Sidak significances for pairwise comparisons both $p < .001$). However, for leptin, the sex by diet interaction that indicated that the diet differences in females were not significant was no longer revealed ($p = .496$). Finally, for CA3 BDNF, the main effect of sex was still revealed in that males had higher CA3 BDNF than females ($\chi2(1) = 7.22$, $p = .007$).

Table AC.1. Summary table of variables in Study 1 with violated homogeneity of variance assumptions, including Levene's test significance values and a summary of model parameters for generalized linear modelling. For these

variables, generalized linear models were conducted to discuss how ANOVA or ANCOVA findings compared.

Study 1 Dependent Variables	Levene's Significance	Generalized Linear Model Summary		
		Specified Distribution (Link Function)	Baseline Model BIC	Generalized Linear Model BIC
Unsupported Rears	$p = .012$	Negative Binomial (Log)	276.474	286.519
Time in Hide Box	$p = .012$	Normal (Identity)	719.194	734.477
Hide Box Entry Latency	$p < .001$	Gamma (Log)	704.367	715.852
Weight Gain	$p = .027$	Gamma (Log)	656.062	548.740
Plasma Leptin	$p < .001$	Gamma (Log)	1145.929	1083.377
CA3 BDNF	$p = .020$	Gamma (Log)	-109.994	-101.831

Note. BDNF = Brain-derived neurotrophic factor.

For Study 2, the variables calories consumed (in the NSFT), food eaten (in the home cage), calories consumed (in the home cage), absolute calorie intake, fasting weight change, plasma ghrelin, and plasma leptin had significant Levene's tests. As with Study 1, these variables (summarized in tabular form below) were analyzed in a factorial generalized linear model design; model summaries are included in the below table (Table AC.2). For calories consumed in the NSFT, there was still a main effect of diet, whereby WD animals consumed more calories than SD animals ($\chi2(1) = 12.40$, $p < .001$). For food eaten in the home cage, there was still a main effect of sex, whereby males ate more food by weight than females ($\chi2(1) = 32.05$, $p < .001$), and still a diet by sex interaction revealing that it was only WD males that consumed more food by weight than WD females ($\chi2(1) = 12.75$, $p < .001$; $p_{sidak} < .001$). However, a further significant difference between diet groups is noted when using Sidak's correction for multiple comparisons (i.e., vs. Tukey's test in the ANOVAs): SD females consumed more food by weight than WD females ($p_{sidak} = .006$). For calories consumed in the home cage, there was still a main effect of sex revealed ($\chi2(1)$

= 32.05, $p < .001$) in that males consumed more calories in the home cage compared to females, along with a significant diet by sex interaction ($\chi2(1) = 12.74$, $p < .001$). Similar to the food weight findings, *post hoc* testing concluded that WD males consumed more calories than WD females ($p_{sidak} < .001$), but WD males and SD males no longer consumed a different amount of calories in the home cage observation (i.e., different from ANOVA, $p_{sidak} = .107$).

To continue with variables from Study 2, for absolute calorie intake findings, there was still a main effect of sex (i.e., males consumed more daily calories than females; $\chi2(1) = 411.90$, $p < .001$), a main effect of diet (i.e., WD consumed more than SD; $\chi2(1) = 34.84$, $p < .001$), and a treatment by diet interaction revealed (i.e., placebo WD animals consumed more calories than placebo SD animals; $\chi2(1) = 9.59$, $p = .002$; $p_{sidak} < .001$). Additionally, the two-way interaction revealed that placebo WD animals consumed more calories than probiotic WD animals ($p_{sidak} = .028$). However, the three way interaction between treatment, diet, and sex was no longer significant ($p = .079$), so in this respect, absolute calorie intake findings did not differ from the relative calorie intake findings and the by cage calorie intake findings. Thus, for calorie intake, the main conclusion that there is only a difference by diet groups (i.e., WD animals consume more calories than SD) in placebo animals and that this difference is mitigated in probiotic-treated animals still stands. Next, for fasting weight loss, results of the generalized linear model again revealed the same finding as the ANCOVA, with a main effect of diet (i.e., SD animals lost more weight in a 24-hour fast than WD animals; $\chi2(1) = 102.52$, $p < .001$). Further, P59 body weight was still a significant covariate in the model ($\chi2(1) = 10.09$, $p = .001$). For the variable ghrelin, generalized linear model findings again reveal the same result as the ANOVA in that there is a significant main effect of sex (i.e., females higher than males; $\chi2(1) = 13.07$, $p < .001$) that is superseded by a significant diet by sex interaction (i.e., SD females higher than SD males; $\chi2(1) = 7.15$, $p = .008$; $p_{sidak} < .001$). Finally, for the variable leptin, findings were not different from ANCOVA results in that there was still a significant main effect of diet (i.e., WD animals had higher leptin than SD animals; $\chi2(1) = 87.73$, $p < .001$), with body weight as a significant covariate in this relationship ($\chi2(1) = 9.21$, $p = .002$).

Table AC.2. Summary table of variables in Study 2 with violated homogeneity of variance assumptions, including Levene's test significance values and a summary of model parameters for generalized linear modelling. For these variables, generalized linear models were conducted to discuss how ANOVA or ANCOVA findings compared.

Study 2 Dependent Variables	Levene's Significance	Generalized Linear Model Summary		
		Specified Distribution (Link Function)	Baseline Model BIC	Generalized Linear Model BIC
Calories Consumed (NSFT)	$p = .018$	Normal (Identity)	393.576	404.640
Food Eaten (Home Cage)	$p < .001$	Gamma (Log)	8.722	3.636
Calories Consumed (Home Cage)	$p < .001$	Gamma (Log)	225.512	219.114
Absolute Calorie Intake	$p < .001$	Gamma (Log)	769.157	647.689
Fasting Weight Change	$p = .003$	Gamma (Log)	587.239	524.670
Plasma Ghrelin	$p = .027$	Gamma (Log)	1119.873	1129.831
Plasma Leptin	$p < .001$	Gamma (Log)	1436.465	1377.616

Note. NSFT = Novelty-suppressed feeding test.

For the 20 hypothalamic inflammatory cytokine proteins measured in Study 2, Levene's test was significant (all *p*s < .05) for all cytokines aside from IL-2 ($p = .100$). For the remaining 19 analytes, generalized linear models with a gamma distribution and log link were conducted. To broadly summarize findings, there was a main effect of treatment revealed by the models for all 19 analytes in that placebo rats were higher than probiotic rats in the following inflammatory cytokines: G-CSF, GM-CSF, GRO/KC, IFN-γ, IL-1α, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, MCP-1, MIP-1α, MIP-3α, RANTES, TNF-α, and VEGF. Additionally, there was a significant treatment by diet interaction revealed (i.e., placebo SD rats were significantly higher than probiotic SD rats) for the following analytes: G-CSF, IL-5, IL-6, IL-10, IL-12p70, IL-17A, MCP-1, MIP-1α, and TNF-α. Of note, IFN-γ was still significantly higher in placebo SD rats compared to

probiotic SD rats. However, it should be noted that even though all analytes were found to be significantly different by treatment or the specified treatment by diet interaction, the model likelihood ratio chi-square statistics (i.e., comparing the fitted model with the baseline model) were only significant for IFN-γ. Thus, IFN-γ being significantly higher in placebo rats fed SD is the meaningful result that persisted through ANOVA analysis and generalized linear model analysis and is the focus of the discussion in Chapter 3.

Overall, there is one difference between ANOVA/ANCOVA and generalized linear model results for Study 1 to discuss. For the variable plasma leptin, the sex by diet interaction that indicated that the diet differences in females were not significant was no longer revealed. This finding indicates that when data is analyzed without the homogeneity of variance assumption being a requirement, then females in the WD and HCD groups do have higher leptin than females in the SD group (i.e., as was revealed in males). For Study 2, differences between findings for hypothalamic cytokine results and benefits and drawbacks to both approaches are summarized in the previous paragraph. As for the remaining variables that were not homogenous in their between-groups variance, no overall differential conclusions were drawn between ANOVA/ANCOVA results and generalized linear model findings. However, as discussed above, there was one additional between-groups difference for food consumed in the home cage interaction (i.e., SD females consumed significantly more food by weight than WD females) when Sidak's test was used *post hoc* to correct for multiple comparisons. In contrast, for calories consumed in the home cage, WD males were not found to consume more calories than SD males when data were analyzed with Sidak's *post hoc* correction. Nevertheless, the main effect of sex (i.e., males consumed more calories than females) and diet by sex interaction (i.e., WD males specifically consumed more calories than WD females) that were revealed by ANOVA were still revealed by generalized linear models. As a whole, ANOVA/ANCOVA findings and generalized linear model findings were highly similar for both studies but it is worth outlining similarities and differences that may occur when different statistical techniques are used in cases of violated statistical assumptions.