PROBIOTICS, PREGNANCY AND THE POSTPARTUM PERIOD: EFFECTS OF DIET AND PROBIOTIC TREATMENT ON MATERNAL AND OFFSPRING HEALTH OUTCOMES IN A LONG-EVANS RAT MODEL

by

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DEDICATION PAGE

I am incredibly fortunate to have many people who deserve to be mentioned here.

To Dan, you've been so supportive and understanding, even when that meant we couldn't see each other for long stretches of time. You have a wonderful gift for comforting and helping me realize when I am being silly.

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ABSTRACT

The Developmental Origins of Health and Disease hypothesis is largely focused on elucidating the mechanisms by which gestational events affect offspring health. This area of research has recently begun examining the interplay between offspring health and the health of those who gestate those offspring. Relatedly, animal and human evidence shows that perinatal administration of certain probiotic strains or gestational diets can impact maternal and offspring health. As such, in a Long-Evans rat model, the studies presented in this dissertation investigated the impact of perinatal nutritional factors on maternal and offspring health-related outcomes. Specifically, maternal and offspring behavioural and physiological variables were examined following maternal administration of the Lacidofil® probiotic (or its placebo) and specific perinatal diet (i.e., Western or standard diet). The first data chapter reports that giving either Western diet or Lacidofil® to mother rats increased certain active maternal care behaviours compared to standard diet and placebo administration, respectively. In the second data chapter, I report marked differences in the maternal cecal content microbiota based on Western diet and Lacidofil® administration. Regarding offspring health, the first study in the third data chapter reports that compared to offspring from placebo-administered mothers, offspring from mothers treated with Lacidofil® weighed more at birth and had higher anogenital distances. Finally, the second study in the third data chapter reports that offspring from Western diet-fed mothers weighed less than offspring from standard diet-fed mothers. Further, offspring from mothers who were given both Lacidofil® and Western diet had higher levels of plasma corticosterone than offspring from mothers given both Lacidofil® and standard diet. Collectively, these studies demonstrate that both the Lacidofil® probiotic and Western diet impacted the measured maternal and offspring health outcomes. Future research should continue examining the potential interactive effects of perinatal nutritional factors on both maternal and offspring health.

LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Percentage
11B-HSD	11beta-hydroxysteroid dehydrogenase
α	Alpha
AA	Arachidonic acid
ABN	Arched-back nursing
ACTH	Adrenocorticotropic hormone
AD	Anogenital distance
ALA	Alpha-linolenic acid
ALDEx2	ANOVA-Like Differential Expression
ANOVA	Analysis of variance
ANS	Autonomic nervous system
BBB	Blood-brain barrier
BCAA	Branched-chain amino acid
BDNF	Brain-derived neurotrophic factor
β	Beta
BMI	Body mass index
cc	Cubic centimetre
CCAC	Canadian Council on Animal Care
CCK	Cholecystokinin
cDNA	Complementary DNA
CFU	Colony-forming units
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GRAS	Generally regarded as safe
GRO/KC	Growth-regulated oncogene keratinocyte chemoattractant
h	Hour
HFD	High-fat diet
γ	Gamma
CORT	Corticosterone
Cq	Quantification cycle
CRF	Corticotropin releasing factor
CV	Coefficient of variation
CVD	Cardiovascular disease
ΔCq	Delta Cq
DHA	Docosahexaenoic acid
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DIO	Diet-induced obesity
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
ΔΔCq	Double Delta Cq
DPP-IV	Dipeptidyl peptidase-4
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nervous system
ER	Estrogen receptor
F	Feeding
F-value	Fisher statistic
g	Grams
G-CSF	Granulocyte-colony stimulating factor
GD	Gestational day
GDM	Gestational diabetes mellitus
GF	Germ-free
HPA	Hypothalamic-pituitary-adrenal axis
HSFD	High-sucrose high-fat diet
IBD	Irritable bowel disease
ICV	Intracerebroventricular
IFN	Interferon
IL	Interleukin
kcal	Kilocalories
LA	Linoleic acid
LC-PUFA	Long-chain polyunsaturated fatty acid
LG	Licking/grooming

LPS	Lipopolysaccharide
M	Mean
M	Molarity
M-CSF	Macrophage colony-stimulating factor
MaAslin2	Microbiome Multivariate Association with Linear Models 2
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
mg	Milligram
MIP	Macrophage inflammatory protein
mL	Millilitre
mm	Millimetre
mPOA	Medial preoptic area
MR	Mineralcorticoid receptor
mRNA	Messenger RNA
n	Sample size
η^2	Eta-squared
NaCl	Sodium chloride
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCD	Non-communicable disease
ng	Nanogram
NGFI	Nerve growth factor-inducible
NGFI-A	Nerve growth factor inducible protein A
nM	Nanomolar
NOD	Non-obese diabetic
NPY	Neuropeptide Y
NSB	Non-specific binding
°C	degrees Celsius
OTU	Operational taxonomic unit
p-value	Probability value
PAAD	Perinatal anxiety and depression
PAI-1	Plasminogen activator inhibitor-1
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PD	Postnatal day
pg	Picogram
PhILR	Phylogenetic Isometric Log-Ratio transform
PICRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PMT	Photomultiplier tube
PPD	Postpartum depression
PVN	Paraventricular nucleus of the hypothalamus
PYY	Peptide YY
q-value	False discovery rate adjusted *p*-value
qPCR	Quantitative polymerase chain reaction
r	Correlation coefficient
r^2	Coefficient of determination
®	Registered trademark
RANTES	Regulated upon Activation Normal T Cell Expressed and Presumably Secreted
RNA	Ribonucleic acid
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RT-qPCR	Reverse-transcription quantitative polymerase chain reaction
SA-PE	Streptavidin phycoerythrin
SCFA	Short-chain fatty acid
SD	Standard deviation
SD	Standard diet
SG	Self-grooming
SOP	Standard operating procedure

SPF	Specific pathogen-free	µL	Microlitre
T2D	Type 2 diabetes	µM	Micrometre
TGF	Transforming growth factor	UniFrac	Unique fraction metric
		VEG-F	Vascular endothelial growth factor
TMB	3,3',5,5'-Tetramethylbenzidine		
		VTA	Ventral tegmental area
TNF	Tumour necrosis factor	W	Water drinking
UCLA	University Committee on Laboratory Animals	WD	Western diet
		× *g*	Times gravity
µg	Microgram		

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CHAPTER 1 INTRODUCTION

MATERNAL CARE

When investigating how internal and external factors influence the mother's health, her behaviour and her offspring's development, laboratory rats are often used to answer these questions (Kristal, 2009). Rats are an altricial mammalian species, meaning that when their offspring are born, they require enormous parental care because they are blind, deaf, and hairless (Kristal, 2009). Rats' maternal behaviour is studied as a representation of altricial species because of their complex but patterned nature (Kristal, 2009). Rat maternal care behaviours include the preparation behaviours of pregnant females, such as nest-building (Denenberg et al., 1969) and self-grooming (Kristal, 2009). Pregnant rats dig into their bedding to build high-sided nests, typically in the corner of their enclosure (Kristal, 2009). To groom themselves, pregnant rats will shift their self-grooming behaviours from their usual routine, such that they spend more time grooming their nipples and urogenital region (Roth & Rosenblatt, 1966, 1967). Once young arrive, rat maternal care behaviours include the behaviours directly associated with caring for the offspring when they are born (Kristal, 2009). Rat dams perform a licking and grooming (LG) behaviour for their offspring to rid them of the afterbirth (Kristal, 2009). They continue to lick and groom the offspring throughout the postpartum period because the neonates cannot urinate or defecate without stimulation from the dam (Kristal, 2009). This licking and grooming behaviour is also essential in restoring the dam's fluid balance (Friedman et al., 1981). Also, licking and grooming is necessary for many aspects of the early development of the offspring (Liu et al., 1997; Champagne et al., 2001).

Another maternal care behaviour important for offspring development is arched-back nursing (ABN), whereby the dam stands over her nest in a kyphotic posture (Stern & Lonstein, 2001). to maximize the number of pups that can nurse simultaneously. Rat dams will nurse, lick, and groom their offspring in bouts (Kristal, 2009) and oftentimes perform these behaviours together.

1.1.1 What is the mechanism for the onset of maternal care?

The onset of maternal care behaviour begins late in pregnancy due to rising estrogen levels and heightened mesolimbic dopamine activity (Rosenblatt, 1994).

The rising estrogen and dopamine activity activate estrogen receptors in the brain, which are crucial for the onset of maternal care (Rosenblatt, 1994). Specifically, for maternal behaviours to occur, estrogen must bind to ER-α receptors in the medial preoptic area of the hypothalamus (mPOA; Champagne et al., 2003). Additionally, the estrogen elevation observed in late pregnancy increases the presence of oxytocin receptor binding (F. Champagne et al., 2001). Oxytocin is critical in modulating maternal licking and grooming (F. Champagne et al., 2001).

Many studies have examined the ability of intracerebroventricular (ICV) administration of oxytocin to induce maternal care behaviours in rats (Pedersen et al., 1994). Conversely, the administration of oxytocin antagonists can prevent the onset of maternal care behaviour in parturient rats (Pedersen et al., 1994). Similarly, if the paraventricular nucleus of the hypothalamus (PVN) or the mPOA is lesioned, initial maternal behaviours are prevented (A. Lee et al., 1999). These findings support the hypothesis that endogenous oxytocin is responsible for the emergence of postpartum

maternal care behaviours (A. Lee et al., 1999; Pedersen et al., 1994). Previous research also found that administering an oxytocin antagonist to the mPOA or the ventral tegmental area (VTA) resulted in increased latencies to retrieve pups (Pedersen et al., 1994). Another study found that when dams characterized as either "high LG-ABN" or "low LG-ABN" were provided with an oxytocin receptor antagonist on the third postpartum day, there were no longer any observable differences in the dams' LG behaviour frequencies (F. Champagne et al., 2001). The results generated by this study provide evidence for increased oxytocin receptors being a mechanism for higher frequencies of LG behaviour that were observed in this group (F. Champagne et al., 2001).

1.1.2 Why is maternal care important to study?

The health status of the mother relates to not only her well-being and how pregnancy will come to affect her, but also feeds into the developmental origins of disease hypothesis (also known as the DOHaD hypothesis or the Barker hypothesis; De Boo & Harding, 2006), meaning that a mother's health status will impact her offspring's future health or lack thereof.

My interest in studying maternal care comes from the large and diverse number of studies that have highlighted the sensitivity of maternal care to both internal and external stimuli, allowing for examination of how these stimuli affect both the mother and her offspring.

Although maternal care has been shown to vary naturally (Champagne et al., 2003), other variations in maternal care are thought to be brought out because of the dam's environment (i.e., external stimuli that she is exposed to; Mashoodh et al., 2009). Through

her altered behaviour, it is hypothesized that this provides environmentally specific programming to her offspring during an early critical period of development Mashoodh et al., 2009), which has been described as the maternal mediation hypothesis (Macrì & Würbel, 2006). As an example of the maternal mediation hypothesis, Korgan and colleagues (2018) found that females mated to males exposed to a chronic high-fat diet (HFD) performed fewer combined LG-ABN behaviours than their control counterparts. This study found that nulliparous females had diminished interest in HFD-exposed males in a partner preference test, and the maternal behaviour exhibited indicated the continuation of that lowered preference towards the offspring (Korgan et al., 2018). Interestingly, if females were mated with HFD-exposed males but housed in an enriched environment during the postnatal period, the effect of male nutritional status on maternal care behaviour was not observed (Korgan et al., 2018).

For internal cues that may affect a mother and how she interacts with her offspring, there are many studies on the effects of maternal nutrition on the postpartum period, including maternal behaviours (Abuaish et al., 2018; Connor et al., 2012; Kougias et al., 2018; Purcell et al., 2011; Smart & Preece, 1973).

Smart and Preece (1973) found that undernourished dams engaged in significantly less pup retrieval and licking/grooming than their control-fed counterparts. This finding could be due not only to the dam's undernourished condition but also to the undernourished status of her offspring (Smart & Preece, 1973). Undernourished dams did not differ in their nest-building behaviours, which do not involve pup contact, suggesting that the offspring may not be as effective in garnering their mother's care (Smart & Preece, 1973). One hypothesis is that their ultrasonic vocalizations may be impaired, thus affecting their ability

to communicate their needs with the dam (Smart & Preece, 1973). This hypothesis is supported by a study investigating undernourished rat dams' care responses to well-nourished versus undernourished offspring, which found that the dams engaged in more licking/grooming behaviours with the well-nourished pups (Smart, 1976). In a more recent but similar study on maternal nutrition effects on behaviour, Connor and colleagues (2012) found that dams fed an HFD engaged in significantly less LG behaviour than controls.

Western Diet

With the introduction of increasingly advanced agricultural and food-processing techniques, humans consume foods altered in one or more of their nutritional characteristics (Cordain et al., 2005). These characteristics include glycemic load, fibre content, micronutrient composition, macronutrient composition, fatty acid composition, acid-base balance, and sodium-potassium ratio (Cordain et al., 2005). Prior to the rapid development of our food infrastructure, foods (including plants and animals) were wild variants and minimally processed (Cordain et al., 2005). As a result, there are several newcomers to modern diets, including dairy products, refined sugars, cereals, and refined vegetable oils (Cordain et al., 2005). Additionally, most salt humans consume comes from processed foods (Webster et al., 2010). Even foods with low glycemic indices, including milk, ice cream and white bread, can negatively affect our hormones (e.g., these foods are insulintropic; (Östman et al., 2001). Diets rich in processed, high-sugar, high-fat, low-fibre foods have been popularized by Western countries, leading such dietary patterns to be known as the "Western diet" (WD; Christ et al., 2019; Imamura et al., 2015; Monteiro et al., 2013).

1.1.3 How does Western diet relate to maternal health and behaviour?

Increasingly, research is investigating dietary patterns and their involvement in health outcomes. In terms of reproductive health, women who consume diets high in red meat, processed and/or sugary foods (i.e., a Western diet) are at risk of pregnancy complications, including the risk of gestational diabetes mellitus (GDM; He et al., 2015; Schoenaker et al., 2015; Zhang et al., 2006) and preeclampsia (Brantsæter et al., 2009; X. Chen et al., 2016).

In addition to affecting maternal health, there is increasing evidence that maternal diet may also affect maternal behaviour. As discussed previously, maternal care behaviours are critical in mammalian early-life development, including for rats, non-human primates, and humans (Bailey & Coe, 1999; F. Champagne et al., 2001; Egeland & Hiester, 1995). Due to the physically demanding nature of the perinatal period, nutrient deficiencies may arise more easily, contributing to an increased risk of postpartum depression (PPD; Sparling et al., 2017). A recent study in women has demonstrated a link between maternal WD consumption and increased risk for PPD (Bolton et al., 2017). Similarly, a more recent study (C. Yang et al., 2021) and a systematic review (Opie et al., 2020) have found links between maternal diet and postpartum symptoms. In a study of Chinese women, researchers found that women experiencing depressive symptoms had lower vegetable intake and an overall decrease in food varieties consumed (C. Yang et al., 2021). The researchers hypothesized that this reduced variety in food consumption may result in lower intake of necessary nutrients such as essential amino acids, minerals and polyunsaturated fatty acids (C. Yang et al., 2021). A systematic review also demonstrated the importance of a good quality diet, demonstrating that women who followed healthy dietary patterns

(e.g., diets rich in vegetables, fruits, legumes, and seeds) had lower scores on PPD measures (Opie et al., 2020).

Compared to healthy diet patterns, WDs are high in branched-chain amino acids (BCAA), which may compete with essential amino acids, such as the precursors to neurotransmitters like serotonin and dopamine, to cross the blood-brain barrier (BBB; Fernstrom, 2005). Serotonin and dopamine availability in the brain are implicated in mood disorders like PPD (Nutt, 2001; Senkowski et al., 2003; Werner & Coveñas, 2010). As previously described, dopamine is crucial in triggering the onset of maternal care behaviours in rodents. Therefore, its availability, or lack thereof, could seriously affect maternal care expression.

There is conflicting evidence about how HFDs and WDs affect maternal care behaviours in rat models. One study found that a maternal HFD (45% fat by kcal; Cat. #D12451 from Research Diets®, Inc.), provided during pregnancy and lactation, negatively impacts maternal care (Connor et al., 2012). It was found that HFD-fed dams do not lick and groom their offspring as much as dams fed a standard chow diet (Connor et al., 2012). Another study employing the same HFD throughout gestation and lactation found that the HFD-fed dams engaged in more licking/grooming and nursing behaviour across seven days compared to dams fed a control chow (15.8% fat by kcal; Cat. #D10012G Research Diets®, Inc.; Kougias et al., 2018). Interestingly, these HFD-fed dams also spent less time away from their pups than the control diet-fed dams (Kougias et al., 2018). In another study, rat dams were given a high-fat diet (60% fat by kcal; Cat. #D12492 from Research Diets®, Inc.) starting on the second gestational day (Purcell et al., 2011). This study performed maternal care observations for the first two postnatal weeks and found no

differences observed during the animals' light phase or the second postnatal week compared to dams fed a standard chow diet (Purcell et al., 2011). However, during the first postnatal week, in the dark phase, HFD-fed dams engaged in more total time spent nursing (i.e., including passive, blanket-posture and arched-back nursing) and more arched-back nursing specifically, compared to standard diet-fed dams. Like the 2018 study by Kougias and colleagues, this study found that HFD-fed dams rested less than their standard diet counterparts (Purcell et al., 2011).

1.1.4 How does Western diet relate to the gut-brain-microbiome axis and inflammation?

The globalization of the WD has led to a rise in non-communicable diseases (NCDs) that are increasingly associated with said diet (Christ et al., 2019; Imamura et al., 2015; Monteiro et al., 2013). A WD can shift the gut microbiota composition, reducing microbial diversity and increasing gut dysbiosis, which ultimately results in inflammation in both rodents and humans (Christ et al., 2019; Z. Hosseini et al., 2016; Lopez-Garcia et al., 2004; Tanoue & Honda, 2012). Persistent, low-grade inflammation is linked with the prevalence of NCDs such as cardiovascular disease, type 2 diabetes mellitus, non-alcoholic fatty liver disease, and some cancers (Canevari & Clark, 2007; Danaei et al., 2013; Z. Hosseini et al., 2016; Kerr et al., 2017; Mozaffarian, 2016). WD administration (Bortolin et al., 2018) and fructose (a common ingredient in Westernized diets; Wang et al., 2020) in rats have also shown to increase serum and plasma pro-inflammatory cytokines levels, respectively.

1.1.5 How does a Western diet affect metabolic markers and stress hormones?

There have been many possible factors associated with maternal diet regarding the effects of diet-induced obesity on the mother's health and that of her offspring (Sullivan et al., 2014). These factors include higher inflammation levels, compromised placental function and hormone dysregulation, including the metabolic hormones leptin and insulin (Sullivan et al., 2014).

The hormone leptin can indicate the metabolic dysfunction that arises due to WD because leptin is responsible for suppressing appetite and storing fat (Elmquist et al., 1998; Figlewicz et al., 2003; Sun et al., 2012). Adipocytes secrete leptin, so as adipose tissue increases due to WD consumption, leptin levels increase, and this can result in leptin resistance (Elmquist et al., 1998; Figlewicz et al., 2003; Sun et al., 2012). It has been shown that leptin is elevated in obese and diabetic mothers, compared to normal pregnancy controls (Hauguel-de Mouzon et al., 2006; Lepercq et al., 1998). Leptin and leptin receptors are involved in activating the stress response via the hypothalamic-pituitary-adrenal (HPA) axis (Roubos et al., 2012), and they can increase inflammatory cytokine levels in the mother (Lappas et al., 2005).

Related to elevated inflammation levels and the stress response, maternal WD is also known to increase the circulation of the stress-related hormone corticosterone (a glucocorticoid; Taves et al., 2011). WDs and HFDs are considered physiological stressors during pregnancy (Connor et al., 2012; Reynolds et al., 2013). These can affect the HPA axis such that basal glucocorticoid levels are heightened (Legendre & Harris, 2006). During pregnancy, to protect the fetus from the effects of glucocorticoids, the placental

enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 is responsible for deactivating corticosterone (Bellisario et al., 2015). However, previous work has demonstrated that maternal HFD lowers 11β-HSD type 2 activity and its expression in the placenta (Bellisario et al., 2015).

1.1.6 Are rodent models of a Western diet translatable to humans?

When studying diet in a preclinical model, it is important to note that laboratory animal diets are a potential issue regarding research translatability (Hintze et al., 2018). Although open-source diets are available, there is still variability due to different mineral content (Finley, 2005) and secondary compounds such as phytoestrogens (Degen et al., 2002; Thigpen et al., 1999). In the United States, the typical "Western" diet is comprised of ~49% carbohydrates, 35% fat and 16% protein, according to the National Health and Nutrition Examination Survey (NHANES; (USDA Agricultural Research Service, 2019).This composition differs from the WD often used in rodent studies (Hintze et al., 2018). The use of diet to induce a disease model in rodents has become popular because, although useful in their way, single gene mutation models such as the *ob* (leptin knockout) and *db* (leptin receptor knockout) models in mice and rats do not parallel the development of obesity in humans (Hintze et al., 2018). Early iterations of the diet-induced obesity model (DIO) in rodents began with HFDs consisting of 82% calories from fat (Lavau et al., 1979; Susini et al., 1979). Although the cafeteria diet has been designed to match Westernized diet patterns better, they are nearly impossible to replicate due to differences in micronutrients (B. J. Moore, 1987). In future studies, there should be a focused effort to increase the translatability of laboratory animal diets to human diets. Some elements that

should be considered include the complex food matrix, food additives and diverse sources of fibre (Hintze et al., 2018).

THE GUT-BRAIN-MICROBIOTA AXIS

In the beginning of DOHaD research, it was demonstrated that low birth weight was a risk factor for adulthood disease (Barker, 1990; Barker, 2004; Barker et al., 1989; Codagnone et al., 2019). Prenatal and early postnatal environment plus the host's genetics can program adulthood health or disease (Barker, 1990; Barker, 2004; Barker et al., 1989; Codagnone et al., 2019). Elements that alter these factors may push an organism toward a later disease state (Codagnone et al., 2019). If multiple factors are affected, this could amplify the extent of the disease state (Barker, 1990; Barker, 2004; Barker et al., 1989; Codagnone et al., 2019). Due to the increasing interest and subsequent research on the gut-brain-microbiota axis, the gut and its microbes are a potential factor in adulthood disease, in addition to prenatal, early postnatal environment and genetics (Barker, 1990; Barker, 2004; Barker et al., 1989; Codagnone et al., 2019).

1.1.7 What is the gut-brain-microbiota axis?

In mammals, the brain, or central nervous system (CNS) is directly connected to the gastrointestinal (GI) tract, also referred to as the "gut," via the vagus nerve (Bravo et al., 2011). Initial research referred to this connection as the "gut-brain axis." However, as gut-brain axis research has increased substantially in recent years, it has become more popular to discuss the "gut-brain-microbiota axis" (see Figure 1.1). The gut-brain-microbiota axis is, therefore, defined as the bidirectional connection between the gut and the brain, in addition to the microbiota inhabiting the gut (Mayer et al., 2022). These

microbes then interact with the brain and the gut via signalling molecules (Mayer et al., 2022). This re-framing of the axis acknowledges that we would not function properly without the estimated trillions of bacteria that inhabit the mammalian gut (D'Argenio & Salvatore, 2015; Mayer et al., 2022). The mammalian gut microbiota comprises not only bacteria, but archaea, fungi, yeasts, viruses, helminth parasites and single-celled eukaryotes, making it an intensely complex environment (D'Argenio & Salvatore, 2015; Margolis et al., 2021).

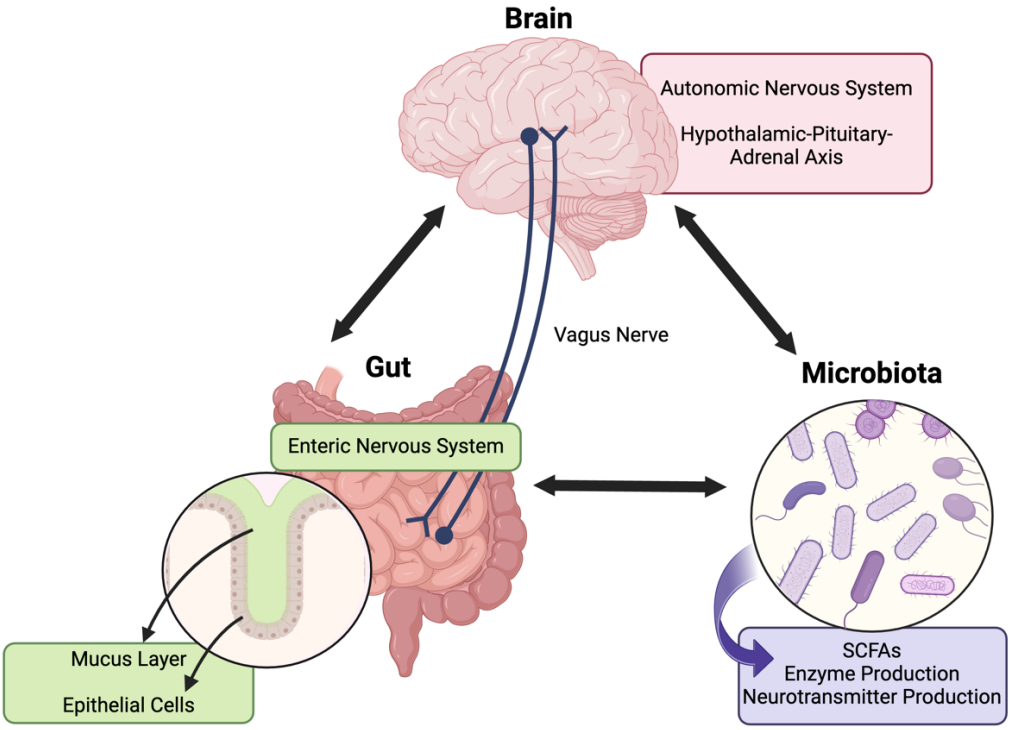

Figure 1.1 A visual summary of the gut-brain-microbiota axis, adapted from figures by Margolis and colleagues (2021) and Mayer and colleagues (2022). Created with Biorender.com.

1.1.8 The importance of microbes demonstrated by germ-free studies

Evidence of the importance of microbiota in everyday functions is apparent in research studies on germ-free (GF) mice (Asano et al., 2012; Sudo et al., 2004). GF mice

show increased hypothalamic-pituitary-adrenal (HPA) axis activity (the primary stress response circuit in mammals) following a stressor and lower brain-derived neurotrophic factor (BDNF) levels in the hippocampus and cortex compared to specific pathogen-free (SPF) mice (Sudo et al., 2004). In a cow's milk allergy model where sensitivity to β-lactoglobulin (a component of cow's milk) was measured, GF mice exhibited increased allergic sensitization to β-lactoglobulin compared to conventional mice (Rodriguez et al., 2011). In another GF study, before the introduction of *Lactobacillus* and *Bifidobacterium* to the gut, GF rats showed slower intestinal transit, compared to after they received a transfer of gut microbes (Husebye et al., 2001).

1.1.9 The functions of the gut-brain-microbiota axis

The gut-brain-microbiota axis aids homeostatic functions (Margolis et al., 2021) such as sleep, immune system regulation and development (Čoklo et al., 2020; Purchiaroni et al., 2013), food intake (Mayer et al., 2022; Osadchiy et al., 2019), enzyme secretion (Čoklo et al., 2020; Flint et al., 2012), and vitamin synthesis (Čoklo et al., 2020; Hill, 1997).

Current evidence suggests that although the vagus nerve does not directly communicate with the microbiota, it is affected by bacterial metabolites and signalling molecules resulting from gut microbiota functions (Margolis et al., 2021). Much like the studies on GF rodents have demonstrated the importance of the microbiome, vagotomy studies illustrate the importance of the vagus nerve in gut-brain-microbiota axis communication (Bravo et al., 2011; Y. Liu et al., 2021; Malick et al., 2015). Gut microbes are crucial in forming signalling molecules, as some of these molecules, for example, short-chain fatty acids (SCFAs), can only be made via fibre fermentation (Mills et al., 2019). In

turn, SCFAs are theorized to affect neurotransmitter production through their influence on enzyme production, which is required for neurotransmitter synthesis (Nankova et al., 2014).

Vagus nerve function can also be modulated by the enteroendocrine and enterochromaffin cells in the gut epithelial lining (Bonaz et al., 2018), as signalling molecules from the microbiome can affect the brain via host circulation or, via gut enteroendocrine cells, enterochromaffin cells, and the mucosal immune system (Walsh & Zemper, 2019). Once the brain receives these signals, its activity can be altered via the autonomic nervous system (ANS) and the HPA axis (C. R. Martin et al., 2018; Yano et al., 2015). The enteric nervous system (ENS) can also directly affect the microbiome by altering the following gut functions: secretion, permeability, immunity, and motility (Margolis et al., 2021).

Within the gut-brain-microbiota axis are the blood-brain and intestinal barriers (Mayer et al., 2022). The permeability of these barriers can fluctuate based on gut metabolites, inflammatory signalling and stress exposure (Mayer et al., 2022). Typically, these barriers prevent inflammatory signalling from reaching the brain (Mayer et al., 2022). The intestinal barrier comprises two main layers: epithelial cells bound by tight junctions (Yu & Yang, 2009) and the mucosal layer composed of mucin protein (Kelly et al., 2015).

Furthermore, due to the importance of the gut-brain-microbiota axis, many (mostly) preclinical studies provide evidence of the microbiome's ability to affect brain function, structure, and development (C. R. Martin et al., 2018; Osadchiy et al., 2019). However, there remain large gaps in knowledge of the gut-brain-microbiota axis mechanisms (Margolis et al., 2021).

1.1.10 Why study the maternal gut-brain-microbiota axis?

In mammals, the gut microbiota is established both during and after birth (Čoklo et al., 2020). The colonization of the gut microbiome at birth is affected by the delivery type, genetics, maternal stress, maternal diet, antibiotic exposure, and many other factors (Margolis et al., 2021).

The composition of the established microbiome is typically stable due to its functional redundancy (Moya & Ferrer, 2016). However, major life events such as pregnancy impact the gut's microbial composition and, therefore, its function (Koren et al., 2012).

The human microbiome changes over the course of pregnancy with concurrent increases in the levels of low-grade inflammation that occur from the first to third trimester (Koren et al., 2012). The observed increase of Proteobacteria in the pregnant microbiome is hypothesized to be involved in the low-grade inflammation observed (Mukhopadhya et al., 2012). Although low-grade inflammation is normal during pregnancy, if there are factors that increase inflammation too much such as gestational diabetes or "leaky gut" this could have serious impacts on fetal growth and increase the risk of preeclampsia (Edwards et al., 2017; Kashtanova et al., 2016; C. J. Kim et al., 2015). Even without changing their diet, pregnant women show differences in metabolic health markers compared to their non-pregnant counterparts, such as increased adiposity, increased leptin, insulin and cholesterol, and increased insulin resistance (Collado et al., 2008). Since diet can further impact these metabolic markers, studying the impact of diet on the pregnant microbiome is important.

1.1.11 How does diet affect the gut microbiome?

The gut microbiome is impacted by many factors, including the host (Bernbom et al., 2006), age (Inoue & Ushida, 2003), environment (Benson et al., 2010; Gacesa et al., 2022) and diet (Connor et al., 2023; David et al., 2014).

Diet composition is a substantial determining factor of the gut microbiome composition and overall gut health (Margolis et al., 2021). In the case of unhealthy diets, these can affect the gut's mucosal layer, resulting in pathogens and commensal bacteria having access to dendritic cells. This can increase gut permeability due to immune activation (Margolis et al., 2021). This diet-induced immune activation is known as metabolic endotoxemia. A review regarding the intake of Western diet-related foods (e.g., foods high in fat, sugar or heavily processed) demonstrated that plasma lipopolysaccharide (LPS) levels were elevated based on these Western foods (André et al., 2019). LPS levels contribute to an organism's inflammation levels (Catorce & Gevorkian, 2016). Due to the inherent inflammation that occurs during a normal pregnancy, any additional factors that may increase inflammation, such as Western diet consumption, can increase the risk of adverse health outcomes for the mother and her offspring (Lana & Giovannini, 2023).

1.1.12 What other factors impact the gut-brain-microbiota axis?

Probiotic research is another area of focus that has been studied in conjunction with the gut-brain-microbiota axis. There is considerable evidence that probiotics provide benefits, including mood-boosting effects (Talbott et al., 2020), reduction in anxiety-like behaviours, the maintenance of important brain metabolites (Lana & Giovannini, 2023), and a reduction in conditions such as antibiotic-associated diarrhea (Hempel et al., 2012)

and irritable bowel syndrome (McFarland & Dublin, 2008). Furthermore, probiotics have demonstrated the potential to mitigate inflammation (Al-muzafar & Amin, 2017) and other health risks associated with Western diet consumption, such as preeclampsia (Brantsæter et al., 2009).

PROBIOTICS

Lactobacilli belong to a large, general bacteria grouping called "lactic acid bacteria", known for their ability to produce lactic acid as a by-product of carbohydrate metabolism (Mayer et al., 2022; Osadchiy et al., 2019). Lactobacilli bacteria are non-spore-forming gram-positive, rod-shaped bacteria (Tannock, 2004). These bacteria prefer to live in areas with high carbohydrate content, including fermented food and drink and inside host organisms, such as mammals (Holzapfel & Wood, 2014). However, carbohydrates are but one of their many nutritional needs (Tannock, 2004). Others include amino acids, peptides, salts, vitamins, and nucleic acid derivatives (Holzapfel & Wood, 2014; Tannock, 2004). Lactic acid bacteria produce short-chain fatty acids (SCFAs; Ljungh & Wadstrom, 2006) as a by-product of their ability to break down otherwise indigestible carbohydrates. SCFAs are capable of many beneficial functions. For example, SCFAs (e.g., acetate, butyrate and propionate) help improve colonic integrity (Yue et al., 2022). They are involved in anti-inflammatory processes, affecting immune cell chemotaxis, cytokine release and reactive oxygen species release (Tan et al., 2014). SCFAs also display antimicrobial activity and improve gut integrity via lowering the pH in the gastrointestinal tract (Tan et al., 2014).

There is a long-standing traditional use of lactobacilli in food products, and as such, the use of these bacteria in the food industry has been investigated thoroughly (Jay, 2000).

The interest in lactic acid bacteria as probiotics began over a century ago with Élie Metchnikoff, a Nobel prize-winning scientist (1845-1916) who proposed that the microbes inhabiting the human gut produced toxins that could affect the host's nervous and vascular systems (Metchnikoff, 1907 as cited by Ljungh & Wadstrom, 2006; Tannock, 2004). Therefore, Metchnikoff deemed gut microbes to cause autointoxication (Tannock, 2004). The theory of autointoxication, that is, the idea of having too many "lower organisms" inhabiting the body that could be potentially poisonous to a person, originally came from the work of Charles Bouchard (Bouchard, 1887; Mathias, 2018).

In opposition to Metchnikoff's extreme idea to surgically remove the large intestine, lest it cause autotoxication, was the idea that any toxin-causing bacteria could be remedied by supplementing the gut microbiota with fermentative bacteria (Tannock, 2004). It was hypothesized that these fermentative bacteria would colonize the gut and provide some benefit to the host (Tannock, 2004). Lactic acid bacteria were proposed for this use because they could ferment milk, preventing it from spoiling (Tannock, 2004). It was thought that if these bacteria could prevent milk from spoiling (i.e., prevent the growth of other bacteria), they may prove beneficial for the gut (Tannock, 2004). Anecdotal proof of the benefits of fermented milk products was taken from the fact that Eastern Europeans had long consumed such products, and some of whom had long lifespans, compared to people from Western Europe (Mathias, 2018; Tamime, 2002; Tannock, 2004).

The definition for probiotics provided by the World Health Organization, updated in 2014 is, "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014, p. 1). The most popular probiotic strains to date are typically from the *Lactobacillus* or *Bifidobacterium* genera (Vera-Santander et al.,

2023), of which the majority are classified as GRAS, or, "Generally Recognized as Safe", since probiotics are low-risk in terms of their infection risk (Ljungh & Wadstrom, 2006). Importantly, in the fast-changing area of probiotic research, a probiotic yeast, S*accharomyces cerevisiae* var. *boulardii*, has yielded impressive abilities to reduce the proliferation of antimicrobial resistance, in both clinical and therapeutic research (Abid et al., 2022). Therefore, it is no longer just bacteria, but now certain types of fungi (i.e., yeasts) that are being investigated for their probiotic potential too. When testing the viability of a probiotic, the foremost important quality it must possess is the ability to survive through the gastrointestinal tract (Ljungh & Wadstrom, 2006). However, data suggest that even dead bacteria travelling through the gastrointestinal tract can confer benefits (Mottet & Michetti, 2005). Many studies favour using multi-strain probiotics over single strains as it is thought that a higher number of strains increases the treatment's efficacy (Ouwehand et al., 2018; Timmerman et al., 2004). However, a recent systematic review investigating the benefits of single- versus multi-strain probiotics found that in most studies, the multi-strain probiotics were no more effective than the single-strain products (McFarland, 2021).

Whether they are used in single or multi-strain applications, lactobacilli are popular probiotic treatment options, as many of their genera are indigenous to the mammalian GI tract (Vera-Santander et al., 2023). As previously described, the lactic acid they produce is beneficial for the GI tract's pH and antimicrobials to combat pathogens by stopping their proliferation and adhesion to the gut lining (Ljungh & Wadstrom, 2006). For example research has found that the probiotic treatment, Lacidofil® (*Lacticaseibacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052), was able to reduce pathogen adhesion (Sherman et al., 2005). Recent work has also demonstrated the potential for lactobacilli to

modulate gut hormones (e.g., glucagon-like peptide-1 [GLP-1], cholecystokinin [CCK] and peptide YY [PYY]; Panwar et al., 2016; Yadav et al., 2013), which may be beneficial for the management of chronic metabolic diseases (Caron et al., 2017). Notably, probiotics are only found in fecal samples while the participant continues to take the product (Tannock et al., 2000). Therefore, it can be implied that continual administration of the probiotic must be maintained to observe the effects.

1.1.13 Lacidofil®

The probiotic treatment used in this collection of studies is Lacidofil®, a dual-strain product comprised of: *Lacticaseibacillus rhamnosus* R0011 (formerly known as *Lactobacillus rhamnosus*; also known as Rosell-11) and *Lactobacillus helveticus* R0052 (also known as Rosell-52; Foster et al., 2011; Tompkins, Barreau, & Broadbent, 2012; Tompkins, Barreau, & De Carvalho, 2012).

The R0011 strain was isolated from a dairy starter culture in 1976 at the Institut Rosell, Inc., in Montréal, Canada (Tompkins, Barreau, & De Carvalho, 2012). The dairy starter culture used for the isolation was made as a dietary supplement (Tompkins, Barreau, & De Carvalho, 2012). R0011 contains the same genes as the *Lactobacillus rhamnosus* GG strain that help promote intestinal epithelial homeostasis through lowering cytokine-induced apoptosis and increasing proliferation (Tompkins, Barreau, & De Carvalho, 2012; Yan et al., 2007).

The R0052 strain was first characterized as *Lactobacillus acidophilus* from its phenotype (Tompkins, Barreau, & Broadbent, 2012). However, DNA-DNA hybridization and sequence typing showed that it was, in fact, *Lactobacillus helveticus* (Tompkins,

Barreau, & Broadbent, 2012). It was first isolated in 1990 at the Institut Rosell, Inc. from a sweet acidophilus milk (Tompkins, Barreau, & Broadbent, 2012). Compared to other *Lactobacillus helveticus* strains, R0052 is unique because it only has one of four cell envelope-associated proteinases, prtH4, and a surface-layer protein (Johnson-Henry et al., 2007). The strain shares 83 genes with some *Lactobacillus acidophilus* strains that are not found in any other sequenced *L. helveticus* strains. These 83 genes include three mucus-binding protein precursors (Tompkins, Barreau, & Broadbent, 2012). Mucus-binding proteins are thought to promote adhesion to the intestinal mucus layer (Klaenhammer et al., 2008).

1.1.14 Probiotic effects on behavioural and molecular markers

As this dissertation will explore further in the studies contained within, probiotic bacteria can alter molecular markers beyond what is contained within the gut.

For example, lactobacilli strains *L. gasseri* ICVB392, *L. reuteri* ICVB395 and *L. gasseri* ICVB396 have been shown to help strengthen epithelial barriers in an *in vitro* study (Belguesmia et al., 2019). These strains were also shown to be capable of modulating GLP-1 and CCK secretion (Belguesmia et al., 2019). Two of the three strains (*L. reuteri* ICVB395 and *L. gasseri* ICVB396) were also able to reduce lipid accumulation in adipocytes (Belguesmia et al., 2019). This lipid-lowering ability has also been demonstrated *in vivo*, in a study with C57BL/6 mice, where *L. bulgaricus* administration reduced adipocyte size in mice fed a high-fat diet (Takemura et al., 2010).

Probiotics interact with the HPA axis, so acute (Ait-Belgnaoui et al., 2012) and chronic probiotic treatments (Foroozan et al., 2021) can alter stress hormone expression in

response to various stressors. A study using partial restraint stress in female Wistar rats in conjunction with a 2-week probiotic regimen (*L. farciminis*) found that the treatment reduced the plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone post-stressor, compared to the levels of the stress-only group (i.e., stress without probiotic supplementation; Ait-Belgnaoui et al., 2012). A chronic high-fat diet model in mice demonstrated the ability of *L. rhamnosus* GG to reduce not only body weight, but serum corticosterone levels and anxiety-like behaviours (Foroozan et al., 2021).

Lacidofil® has also been used to help counteract the negative effects of generational stress experienced by rat offspring due to maternal separation (Callaghan et al., 2016). The administration of Lacidofil® to either the F1 infant male rats or the F0 stress-exposed fathers resulted in the extinction of conditioned aversive associations (Callaghan et al., 2016). The authors propose that, although there are many ways that stress can be transmitted through generations (e.g., maternal care behaviours, epigenetic markers) their results suggest that the effects of stress on the microbiota may also be a contributing factor, hence why probiotics ameliorate the effects of generationally inherited stress in this study (Callaghan et al., 2016). In a study investigating early-life stress using a maternal separation protocol in Sprague-Dawley rats, researchers found that maternal administration of Lacidofil® throughout the separation protocol prevented lasting fear memories due to a fear conditioning protocol (Cowan et al., 2016). Several studies demonstrate that postnatal stressors can accelerate puberty in both female rodents and adolescent girls (Davis et al., 2020; Strzelewicz et al., 2019; Tremblay & Frigon, 2005). In male rodents, one study found delayed puberty onset (Davis et al., 2020), unlike the accelerated onset observed in females (Davis et al., 2020; Strzelewicz et al., 2019).

There are also preliminary linkages between probiotic consumption throughout pregnancy and a lowered risk of preterm birth, preeclampsia and gestational diabetes mellitus (GDM) - all of which are more likely to occur in overweight or obese patients (Brantsaeter et al., 2011). In turn, GDM increases the risk of offspring developing metabolic syndrome or diabetes in adulthood (Dabelea, 2007). In a study where mothers were given *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 at a dosage of 10^{10} colony-forming units (CFU) per day from the first trimester through the cessation of exclusive breastfeeding, the group that received the probiotic treatment in addition to dietary counselling had a significant decrease in GDM risk (Luoto et al., 2010).

My previous research found that Lacidofil® increased maternal arched-back nursing and licking/grooming behaviours when dams were provided with an acute treatment for six weeks (O'Leary, 2019). Although I have not explored possible mechanisms for this effect, there are a few possibilities. In addition to synthesizing signalling molecules, the gut microbiome also affects host hormone levels (Baker et al., 2017).

Firstly, lactic acid bacteria have been studied for their indirect links to increased oxytocin production (Poutahidis et al., 2013). In a 2013 study by Poutahidis and colleagues, they demonstrated that probiotic administration (*L. reuteri* ATCC-PTA-6475) was capable of up-regulating oxytocin levels in the plasma of mice after they received a skin biopsy (Poutahidis et al., 2013). This effect was abolished by performing a vagotomy, illustrating the connection between the probiotic delivery to the gut and the vagal nerve connection to the CNS (Poutahidis et al., 2013).

Secondly, as discussed previously, the hormones oxytocin and estrogen are crucial for expressing maternal care behaviour (Pedersen et al., 1994; Rosenblatt, 1994). Studies have shown that the gut microbiome can modulate estrogen levels by producing the enzyme β-glucuronidase (Plottel & Blaser, 2011). In the bloodstream, estrogens can be bound to proteins or free (Kwa et al., 2016). The liver is responsible for conjugating estrogens (and estrogen metabolites) via glucuronidation or sulfonation so that these products can be excreted in bile, urine or feces (B. T. Zhu & Conney, 1998). β-glucuronidase in the gut (Cole et al., 1985) can deconjugate estrogens excreted in bile (Kwa et al., 2016). This deconjugation renders the estrogens capable of reabsorption into the bloodstream, where they can continue exerting their effects (Kwa et al., 2016). The production of the enzyme β-glucuronidase by lactic acid bacteria (Cole et al., 1985), such as the strains within Lacidofil®, could also be linked to maternal care onset since β-glucuronidase helps metabolize estrogen (Plottel & Blaser, 2011). Any influence on the estrobolome (i.e., the collective of estrogen-metabolizing producing bacterial genes), such as these enzymes, could, therefore, impact maternal care expression (Numan et al., 1977; Plottel & Blaser, 2011).

Probiotic treatments may also provide benefits when Western diets are involved. In one study, an acute multi-strain probiotic treatment improved liver function in rats fed a high-sucrose/high-fat diet (HSFD) and lowered their plasma leptin levels (Al-muzafar & Amin, 2017). The acute probiotic treatment helped slow weight gain and restore glucose-insulin homeostasis (Al-muzafar & Amin, 2017). While HSFD-only rats showed increased serum levels of tumour necrosis factor (TNF)-α and interleukin (IL)-6, compared to the control diet rats, the HSFD-fed rats that were provided with an acute probiotic treatment

showed lowered levels of these same inflammatory factors, compared to the HSFD-only rats (Al-muzafar & Amin, 2017).

There is great interest in probiotic treatment during pregnancy and postpartum for their benefits on maternal and offspring health. Current evidence suggests that probiotics are most effective when started during pregnancy, compared to the postnatal period (Berti et al., 2017). The benefits of probiotic use during pregnancy range from maintaining serum insulin levels (Asemi et al., 2012), to lowering the risk of severe preeclampsia (Brantsaeter et al., 2011).

OFFSPRING STRESS AND METABOLIC PROGRAMMING

Organisms change throughout their lifetime based on their genetics, epigenetics, and the environment (Murgatroyd & Spengler, 2011). However, there are critical periods of development, beginning with the prenatal and continuing into the postnatal period, where an organism is susceptible to its environment, which includes stressors that may be present (Murgatroyd & Spengler, 2011). During these critical periods, stress can affect the organism, making it more susceptible to stress-related disease in adulthood (Murgatroyd & Spengler, 2011).

1.1.15 The Hypothalamic-Pituitary-Adrenal Axis

Stressors can be psychological, physiological, tangible, or perceived (De Kloet et al., 1998). The HPA axis is the primary stress response circuit in mammals, and it comprises structures and tissues within the central nervous system and periphery (S. M. Smith & Vale, 2006). Within the central nervous system, the paraventricular nucleus of the hypothalamus (PVN) synthesizes and releases corticotropin-releasing factor (CRF), which

signals the anterior pituitary gland to secrete ACTH into circulation (Rivier & Vale, 1983). Once ACTH is circulating, it signals the adrenal glands in the periphery (the adrenal cortex specifically) to synthesize and secrete glucocorticoids (S. M. Smith & Vale, 2006). When released into circulation, glucocorticoids can create physiological and behavioural changes, allowing the organism to respond to the stressor (Bamberger et al., 1996; Munck et al., 1984).

The main glucocorticoid expressed in rodents is corticosterone; in humans, it is cortisol (S. M. Smith & Vale, 2006). At basal levels, corticosterone aids in maintaining circadian rhythms, energy homeostasis and attention (De Kloet et al., 1998). Glucocorticoids, such as corticosterone, bind to glucocorticoid receptors (GRs), which are prevalent in the brain and peripheral tissues (S. M. Smith & Vale, 2006). When glucocorticoids bind to GRs, they inhibit the HPA axis' response to stress, thus creating a negative feedback loop (S. M. Smith & Vale, 2006). Notably, glucocorticoids have a high affinity for and preferentially bind to mineralocorticoid receptors (MRs; (De Kloet et al., 1975; S. M. Smith & Vale, 2006; Veldhuis et al., 1982). Due to glucocorticoids' low affinity for GRs, they only bind to GRs when their levels are high (e.g., when responding to a stressor or at a circadian peak; (De Kloet et al., 1998; Reul & De Kloet, 1985). Therefore, glucocorticoids in basal levels tend to bind to MRs mainly (S. M. Smith & Vale, 2006). Conversely, when an organism reacts to a stressor and has elevated glucocorticoid levels, the glucocorticoids bind to GRs to assist with stress recovery (De Kloet et al., 1998; Smith & Vale, 2006). Two crucial feedback sites for glucocorticoids in the brain have heightened expression of GRs: the paraventricular nucleus of the hypothalamus and the hippocampus (S. M. Smith & Vale, 2006). The hypothalamus is also vital for regulating energy

homeostasis (S. M. Smith & Vale, 2006). Specifically, neurons within the arcuate nucleus of the hypothalamus are sensitized to the circulation of metabolic hormones, insulin and leptin (Schwartz et al., 1992, 1996). Crucially, the expression of GRs in the hippocampus is programmed via maternal care behaviours early in the postnatal period, and these changes in expression have enduring effects on how the HPA axis responds to stressors (De Kloet et al., 1998; Weaver et al., 2004).

1.1.16 Maternal Care Programs the HPA Axis

In rodents, maternal care behaviour modulates the offspring's stress response programming through gene expression alterations in the brain (Weaver et al., 2004). The offspring of rat dams that provide high amounts of LG-ABN behaviours have heightened GR expression in the hippocampus. Subsequently, these offspring are more sensitive to glucocorticoid feedback (Weaver et al., 2004). The effects of maternal LG-ABN behaviours have been studied both *in vivo* and *in vitro*. The results of these studies suggest that the observed increase in offspring GR gene expression is due to heightened serotonin activity (Laplante et al., 2002; Meaney et al., 2000; Weaver et al., 2001). With the increased serotonin activity at its receptors, there is also an increase in the expression of nerve growth factor-inducible protein A (NGFI-A) within the hippocampus (Weaver et al., 2004). Within the non-coding exon 1 of the GR gene, a promoter region, exon 1_7, has a binding site for NGFI-A (McCormick et al., 2000). In a Long-Evans rat maternal care model that grouped dams into "high LG-ABN" and "low LG-ABN" caregivers, researchers found that in the hippocampal tissue of offspring, the offspring of low LG-ABN mothers always had the

same specific methylation sites within the exon 1_7 GR gene promoter sequence (Weaver et al., 2004).

Conversely, in offspring from high LG-ABN dams, this promoter sequence was rarely methylated, and there is increased expression of GR with the exon 1_7 sequence (Weaver et al., 2004). This evidence suggests that the exon 1_7 sequence increases GR gene expression due to active maternal care (Weaver et al., 2004). Interestingly, if pups were cross-fostered to a dam that had the opposite maternal care pattern (e.g., a pup from a low LG-ABN dam was fostered to a high LG-ABN dam), the offspring displayed the methylation pattern associated with their cross-fostered dam (Weaver et al., 2004).

1.1.17 Maternal Influences on Early-Life Metabolic Programming

Like the stress response, an organism's metabolism is also programmed early in life. Metabolic programming occurs through inhibition of the anorexigenic pathway, activation of the orexigenic pathway, (Davidowa et al., 2003, 2006) and differential exposure to neuropeptides (e.g., neuropeptide Y) and metabolic hormones (e.g., insulin and leptin; Srinivasan & Patel, 2008).

Leptin is a metabolic hormone and neurotrophic factor whose primary neural activity occurs in the arcuate nucleus of the hypothalamus (Bouret, 2010). Leptin is produced by the *ob* gene, secreted by adipocytes, and is thus involved in food intake and metabolic functions (Havel, 1998). Besides being synthesized in adipocytes, leptin can be found in the gut mucosa (Bado et al., 1998; Cinti et al., 2000), placenta (Masuzaki et al., 1997), mammary glands and breast milk (Casabiell et al., 1997; Houseknecht et al., 1997).

Maternal nutrition not only has ramifications for the mother's health status but also affects her milk composition, which has programming power over her offspring's metabolism. Breast milk is the ideal infant nutrition form (Jeurink et al., 2013). For example, in humans, during the first six months of the infant's life, it is best if they can be fully breastfed, as evidence suggests that full breastfeeding provides greater protection against gastrointestinal infection compared to partially breastfed infants (M. S. Kramer et al., 2003).

Within the DOHaD framework, *in utero* and early-life events are considered key development periods when it is possible to program an organism's future phenotype (Hanson & Gluckman, 2014). This inherent plasticity of early life is a natural target for interventions and increased understanding of programming mechanisms because there are serious implications for the organism's fitness (Berti et al., 2017). As previously discussed, concerning the gut-brain-microbiota axis, one such target is the maternal microbiome. The maternal microbiome offers immense programming power in the early life of her offspring. In addition to the maternal gut microbiota, offspring are exposed to the maternal vaginal and (Dominguez-Bello et al., 2010) breast milk microbiota too (Pannaraj et al., 2017). Therefore, understanding the implications of the maternal microbiome and metabolite transfer via breast milk could provide insight into offspring metabolic programming (Berti et al., 2017).

Sufficient nutrition during pregnancy is necessary for the energy and nutrient demands placed on the mother, which help support fetal growth, including gene expression, neural development, immune development, hormone secretion and gut microbiome modulation (Berti et al., 2016; Cetin et al., 2010; Collado, Cernada, et al., 2012). Maternal

diet is also important in breast milk composition (Innis, 2007; Krešić et al., 2013; Urwin et al., 2012). Importantly, the fatty acids within breast milk are sensitive to diet (Innis, 2007; Krešić et al., 2013; Urwin et al., 2012). Long-chain polyunsaturated fatty acids (LC-PUFAs), such as docosahexaenoic and arachidonic acids (DHA and AA, respectively), are crucial for neonatal growth and development (Bernardi et al., 2012; Krešić et al., 2013). Linoleic and alpha-linoleic acids are precursors for LC-PUFAs, and the primary source of these acids in maternal milk is the maternal diet (Agostoni, 2010; Innis, 2007; Krešić et al., 2013). With typical Western diets, it was observed that milk composition shifted starting in the 1990s, such that there was a decrease in DHA and an increase in linoleic acid (LA), lowering the total amount of n-3 fatty acids in milk, which can negatively affect infant health (Krešić et al., 2013). Therefore, it is important to consider the impact of diet on milk composition to ensure the increased presence of LC-PUFAs (Krešić et al., 2013). A study of Croatian women found that maternal dietary DHA intake strongly correlated with her milk's DHA content (Krešić et al., 2013). These results are consistent with other women who adhere to a Mediterranean diet, consisting of moderate carbohydrate intake and a higher intake of fats (Antonakou et al., 2013). Milk composition changes are also observed in diet studies in animals. Western diets delivered to rats throughout pregnancy and lactation increase milk lipid (Del Prado et al., 1997) and lactose concentrations (Y. Chen et al., 2017).

Like diet, probiotics have also been shown to impact milk composition. In humans, it has been found that a multi-strain probiotic increases the abundance of the oligosaccharides found in human milk (Seppo et al., 2019). Another study in humans combining fatty acid supplements and a probiotic found that participants receiving both

fatty acids and probiotics had higher γ-linolenic acid concentrations (which may help treat inflammation; Hoppu et al., 2012; Sergeant et al., 2016). A study in rats demonstrated that probiotic-treated dams had higher transforming growth factor (TGF)-β1 levels in their milk compared to controls, which has implications for the milk's ability to bolster the immune system (Messias et al., 2019). A study showed that administering a probiotic during pregnancy and lactation increased TGF-β2 levels in human milk (Rautava et al., 2002). Notably, it has been found that maternal obesity can impact immunoregulatory factors, including TGF-β2 in human milk (Collado, Laitinen, et al., 2012; Rautava et al., 2002). In dams fed a high-fat diet (HFD), there were a few differences in milk composition at postnatal day 21, such that the HFD-fed dams produced milk with a higher protein content (Purcell et al., 2011). Interestingly, there was no difference in milk sugar content between HFD and control-fed dams (Purcell et al., 2011).

1.1.18 Probiotic Treatments Affect Milk Composition

Various probiotic treatments have demonstrated the ability to influence the composition of breast milk, including the modulation of γ-linoleic fatty acid (GLA) and TGF-β (Hoppu et al., 2012). Probiotic bacteria such as lactobacilli produce metabolites, including linolenic acid (Alonso et al., 2003; E. S. Hosseini et al., 2015), an essential omega-3 fatty acid crucial in neural development (Chang et al., 2009).

Fortunately, many probiotic studies have been conducted in pregnant women, showing no significant side effects to the mother or child (Reid et al., 2013). Some probiotic strains have been approved for infant formula, as studies demonstrate an immune-boosting benefit of specific strains (Holscher et al., 2012). In a rodent study, probiotics increased

milk protein content (Messias et al., 2019). In a study by Ma and colleagues (2020), three probiotic strains (*S. cerevisiae* ACCC 20065, *B. subtilis* ACCC 11025 and *E. faecalis* CICC 23658) were studied as single strains and as a combination probiotic in lactating goats. It was found that the combination probiotic significantly increased milk fat, whereas the *S. cerevisiae* and *B. subtilis* single strains, in addition to the combination probiotic, increased the protein content (Ma et al., 2020). Only the *S. cerevisiae* and *B. subtilis* treatment groups significantly increased milk lactose (Ma et al., 2020). Interestingly, the *E. faecalis* treatment group had the least milk fat fluctuation over eight weeks (Ma et al., 2020). Researchers hypothesize that the observed increase in milk fat could be due to improved fibre digestibility because of the combination probiotic (Ma et al., 2020).

Probiotics may interact with the fatty acids found in breast milk, as they may recruit similar signalling pathways (Kankaanpää et al., 2004; Laitinen et al., 2006). In a study using *Lactobacillus rhamnosus* GG and *Bifidobacterium lactic* Bb12, participants took these probiotic strains daily throughout pregnancy, including one month postpartum (Hoppu et al., 2012). Participants also received dietary intervention via nutrition counselling (Hoppu et al., 2012). In the group that received both dietary counselling and probiotic treatment, it was found that these participants had greater levels of γ-linolenic acid in their breast milk compared to the diet intervention/placebo group (Hoppu et al., 2012). The modulation of dietary fats provided by nutrition counselling, with the addition of a daily probiotic, seems to promote the increase in γ-linolenic acid in the milk (Hoppu et al., 2012).

Summary of Dissertation Goals

Due to the wide-ranging impact of the gut-brain-microbiome axis, my overarching goal for my dissertation was to investigate several outcomes that both probiotics and diet are known to affect in mothers and their offspring (see Figure 1.2). Therefore, I split my dissertation into three data chapters, each exploring a different area.

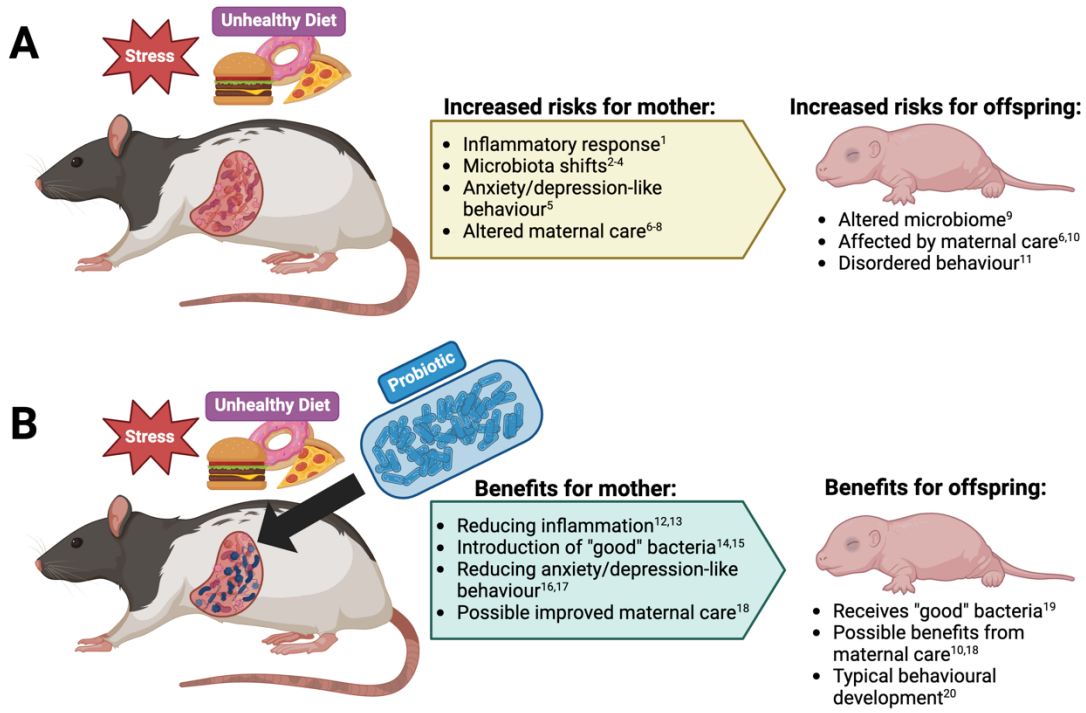

Figure 1.2 An illustration of the A) possible risks of stressors, such as an unhealthy, "Western" diet affecting the mother and her offspring and B) the possible benefits that probiotic administration has that may counteract or mitigate the effects of an unhealthy "Western" diet (1 - Walker et al., 2012; 2 - Collado et al., 2008, 3 - Collado et al., 2010; 4 - Santacruz et al., 2010; 5 - Slykerman et al., 2017; 6 - Connor et al., 2012; 7 - Kougias et al., 2018; 8 - Purcell et al., 2011; 9 - Steegenga et al., 2017; 10 - Weaver et al., 2004; 11 - Gawlińska et al., 2021; 12 - Al-muzafar & Amin, 2017; 13 - Asemi et al., 2012; 14 - Foster et al., 2011; 15 - Krishna et al., 2015; 16 - Bravo et al., 2011; 17 - Rackers et al., 2018; 18 - O'Leary, 2019; 19 - Ma et al., 2020; 20 - Cowan et al., 2016). Created with Biorender.com.

For Chapter 2, I wanted to continue researching how rodent maternal care is impacted by probiotic administration. I wanted to explore how maternal diet (standard lab chow versus Western diet) and probiotic administration (Lacidofil®) may interact to impact maternal care behaviour and maternal outcomes, such as corticosterone and inflammation, which are known to be impacted by both diet and probiotics.

For Chapter 3, I wanted to investigate maternal microbiome composition and how it is affected by probiotics alone and subsequently in conjunction with Western diet administration. To accomplish this, Chapter 3 contains two separate studies using two cohorts of female Long-Evans rats.

Lastly, for Chapter 4, to determine if there were any offspring programming effects due to maternal probiotic administration or maternal diet administration in conjunction with maternal diet, offspring developmental outcomes, including corticosterone levels and glucocorticoid receptor expression, in addition to maternal milk leptin, were studied. Similarly to Chapter 3, Chapter 4 contains two separate studies. The first study was exploratory in nature to confirm that maternal probiotic administration does not impact offspring anogenital distance at birth and to determine if I could collect a sufficient quantity of breastmilk to detect the metabolic hormones leptin and insulin via enzyme immunoassay. The second study examines how maternal probiotic and diet administration impacts offspring stress and metabolic programming by measuring mRNA expression of GRs in offspring hippocampal tissue, plasma corticosterone and plasma leptin in offspring.

CHAPTER 2 THE EFFECTS OF DIET AND PROBIOTIC ADMINISTRATION THROUGHOUT THE PERINATAL PERIOD ON MATERNAL BEHAVIOUR, INFLAMMATION AND METABOLIC HORMONES

INTRODUCTION

There is growing evidence that the maternal microbiome and efforts to improve it have potentially important implications for maternal health (e.g., maternal metabolism; Asemi et al., 2012; Laitinen et al., 2008), as well as pregnancy outcomes (Brantsaeter et al., 2011; Lindsay et al., 2013; Luoto et al., 2010). Due to the numerous changes (e.g., hormonal, physical, emotional) that occur during the perinatal period (i.e., the beginning of the pregnancy up until one year postpartum), this is a particularly susceptible time for maternal health (Biaggi et al., 2016; Fisher et al., 2012; Silva et al., 2019). Therefore, probiotics are being studied as a nutritional supplement to maximize maternal health and well-being during the perinatal period.

The oral supplementation of probiotics during pregnancy is generally considered safe, as the risks of bacteria and fungemia are low and unlikely to affect the fetus (Elias et al., 2011). The dangers of systemic absorption are low in healthy populations, and there is no current evidence to suggest that probiotics taken during pregnancy result in poor health outcomes for mothers or their children (Elias et al., 2011). Probiotic treatment during pregnancy may be preventative against multiple health conditions, including gestational diabetes mellitus (GDM; Chen et al., 2020) mastitis (Fernández et al., 2016), constipation (Mirghafourvand et al., 2016), postpartum depression (PPD; Slykerman et al., 2017) and the growth of harmful bacteria such as Group B *Streptococcus* (Ho et al., 2016; Reid et al.,

2013; Sheyholislami & Connor, 2021). Despite the vast array of possibilities for probiotic use during pregnancy and lactation, their effectiveness in treating pregnancy-related issues is uncertain (Sheyholislami & Connor, 2021). A recent meta-analysis of probiotic use during pregnancy found that most reported adverse events are due to maternal gastrointestinal upset (Sheyholislami & Connor, 2021). Probiotic use in pregnancy also demonstrates the benefits of lower inflammation (Asemi et al., 2012; Sheyholislami & Connor, 2021), decreased infection risk (Othman et al., 2007; Sheyholislami & Connor, 2021) and better glucose metabolism (Sheyholislami & Connor, 2021; J. Zheng et al., 2018). Since it is often populations with underlying health conditions that suffer from increased risk of infection and higher inflammation, studies should include pregnant populations with obesity, allergic disease, and bacterial vaginosis, as probiotics may be especially beneficial to these populations (Sheyholislami & Connor, 2021).

There is currently a dearth of clinical studies looking into the benefits of probiotic use during pregnancy; however, current research from preclinical models (e.g., *in vitro* and *in vivo*) supports the progression of this research into clinical trials (Reid et al., 2013). For example, the probiotic bacteria *Lactobacillus rhamnosus* has been studied for its anti-inflammatory and corticosterone-reducing abilities in mice, in addition to reducing anxiety- and depression-like behaviours (Bravo et al., 2011; Rackers et al., 2018). These effects could greatly benefit the perinatal period due to the increased levels of perceived stress, which has been shown to increase as the period goes on (Long et al., 2023). It is also worth noting that improved maternal nutrition can reduce the risk of infants developing disease in adulthood (e.g., cardiovascular and metabolic disease; Reid et al., 2013). In

addition to their benefits on the host microbiome, probiotic bacteria can degrade toxins, thus reducing host uptake (Reid et al., 2013).

2.1.1 Maternal Mental Health and Probiotics

Between 10-20% of all pregnant women experience anxiety and depressive symptoms (Heron et al., 2004; Marcus et al., 2003). These symptoms often go untreated, however, due to waitlists at medical centres, inaccessibility, and fears of using medication while pregnant (Einarson et al., 2010; Kopelman et al., 2008). There is preliminary evidence that probiotics could benefit people who are suffering from increased anxiety and depressive symptoms (Nadeem et al., 2019). More specifically, probiotic use for such symptoms has been studied in pregnant women, with results showing that perinatal probiotic administration reduced maternal depression and anxiety symptoms (Barthow et al., 2016; Slykerman et al., 2017).

2.1.2 Maternal Health and Western Diet

Nutritional effects during pregnancy, such as probiotics, can be positive, but specific dietary patterns may harm maternal health. Notably, there is an association between maternal Western diet consumption and an increased risk for postpartum depression (Bolton et al., 2017) and GDM (Zadeh et al., 2020).

PPD has many risk factors, including pregnancy complications (Biaggi et al., 2016), hormonal changes (Schiller et al., 2015), and possibly consuming highly processed, high-fat and high-sugar foods (i.e., a Western diet; Baskin et al., 2015; Pina-Camacho et al., 2015). However, there is currently little research on how much these disorders are affected

by diet and nutritional supplements (Silva et al., 2019). Due to the possibility that dietary patterns can change during pregnancy (Di Simone et al., 2020; Gardner et al., 2012), it is important to research how diet can impact maternal health. A systematic review of perinatal anxiety and depression (PAAD) and dietary patterns was inconclusive on the association between Western diet and PAAD (Silva et al., 2019). As there are significant implications for maternal and fetal health, further research should be conducted to determine the magnitude of health risk(s) posed by Western diet consumption (Silva et al., 2019).

PPD affects both the mother and child, as it leads to an increased likelihood of subsequent depressive episodes (Cooper & Murray, 1995). If maternal depressive episodes occur, children are affected by the resulting compromised mother-child interactions (Murray et al., 1996). Pregnancy also brings hormonal changes that can impact maternal mental health (Skalkidou et al., 2012). For example, there is a link between oxytocin (a hormone essential for attachment and maternal behaviour) and depression, such that the mediation of postpartum oxytocin levels could be a potential factor in improving PPD (H.-J. Lee et al., 2009; Moura et al., 2016).

In addition to PAAD and PPD, GDM has many adverse outcomes for mothers as well as their infants (Barthow et al., 2016). When mothers consume a Western diet, their risk of developing GDM is increased (Sedaghat et al., 2017). For mothers, GDM can result in short-term adverse outcomes such as gestational hypertension, preeclampsia, cesarean delivery, and death (Poston et al., 2011). GDM also has long-term implications for maternal health, including the risk of developing type 2 diabetes and cardiovascular disease (Vohr & Boney, 2008). Therefore, metabolic markers must be investigated in studies involving maternal diet. Leptin is a key metabolic hormone in the central nervous system (Bouret,

2010), signalling food intake behaviour (Havel, 1998). If leptin signalling or leptin receptors become compromised, this can lead to metabolic dysregulation and obesity (Zhou & Rui, 2013). During pregnancy, leptin levels increase as the placenta produces leptin, contributing to the overall higher leptin levels observed during this time (Pérez-Pérez et al., 2020). This increase in circulating maternal leptin due to the placenta can potentially worsen insulin resistance and instances of GDM (Pérez-Pérez et al., 2020).

2.1.3 Maternal Microbiota

In conjunction with hormonal changes, microbiota changes during pregnancy have also been reported (Di Simone et al., 2020; Koren et al., 2012). During pregnancy, the increased estrogen and progesterone levels impact the maternal gut microbiome (García-Gómez et al., 2013) such that bacterial diversity (beta diversity) increases, but bacterial richness (alpha diversity) decreases (Koren et al., 2012).

The gut microbiome helps regulate circulating estrogens (Baker et al., 2017; Flores et al., 2012); therefore, microbes that help balance naturally occurring estrogen changes may benefit the host (Rackers et al., 2018). For example, certain microbes secrete β-glucuronidase (β-gluc), an enzyme that activates estrogen (Baker et al., 2017). As such, these microbes could be beneficial in the postpartum period, which is characterized, in part, by low estrogen levels (Baker et al., 2017).

With growing gut-brain-microbiota axis research, there are more studies investigating how a dysregulated gut microbiome may be linked with numerous psychological illnesses, including anxiety and depression (Dinan & Cryan, 2017). As the microbiota is involved in both the regulation of the central nervous and immune systems,

pregnancy-induced microbiota shifts could affect depression or anxiety onset (Rackers et al., 2018; Sharon et al., 2016). Further, the gut microbiome controls serotonin synthesis and secretion (Desbonnet et al., 2015; Kelly et al., 2015), with low levels of serotonin being associated with depression and anxiety (Deakin, 1998).

Many studies have shown that nutritional interventions have an impact on anxiety and depressive behaviours either in the offspring (Krishna et al., 2015) or in the mothers themselves (Slykerman et al., 2017). A study in Wistar rats exposed to a neonatal inflammatory stress response via lipopolysaccharide (LPS) showed altered maternal care behaviours as adults (Walker et al., 2012). This study clearly shows the importance of investigating diets capable of inducing similar inflammatory responses (e.g., a Western diet) or microbiota shifts that may increase the risk of maternal anxiety or depression. Furthermore, there is evidence that shifts in the maternal gut microbiome across pregnancy may be exacerbated if the mother is overweight (Collado et al., 2008, 2010; Santacruz et al., 2010).

2.1.4 Maternal Health and Probiotics

Since pregnancy is a time of intense health demands on mothers and great programming power concerning offspring health outcomes, probiotics are regarded as an important therapeutic manipulation that may benefit both the mother and her infant (Lindsay et al., 2013). Investigation into the benefits of probiotics is warranted, as women are averse to taking medication during the perinatal period due to reservations regarding potential side effects and the possibility of transfer via breastmilk to their child (Boath et al., 2004; Chabrol et al., 2004; Whitton et al., 1996). Therefore, there is a need for more

research into probiotic administration for maternal metabolism and pregnancy outcomes to determine their viability and benefits as perinatal supplements (Lindsay et al., 2013).

Probiotic supplementation during pregnancy may provide some protection against the development of GDM. In addition to dietary counselling for GDM, probiotic administration is associated with improved outcomes in mothers (Laitinen et al., 2008; Luoto et al., 2010). For example, there are promising results of probiotics lowering the instances of GDM in a study that utilized a probiotic supplement in conjunction with a diet counselling intervention (Luoto et al., 2010). The probiotic-treated counselling group had the lowest instances of GDM (Lindsay et al., 2013; Luoto et al., 2010). Interestingly, a meta-analysis found that GDM was a significant risk factor for PPD (Zhao & Zhang, 2020). However, the mechanism(s) that increase PPD risk due to GDM are unclear (Zhao & Zhang, 2020). The stress from having GDM itself may be a risk factor for PPD (O'Hara & McCabe, 2013), as there is a commonality between GDM, depression onset and insulin resistance (Hinkle et al., 2016; Kan et al., 2013). Other common factors could include increased inflammatory cytokines and hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Pariante, 2017; Robakis et al., 2017). There is also a positive association between pre-pregnancy obesity and PPD (Steinig et al., 2017). Obesity may increase stress and inflammation, which coincides with the risk factors for depression (Ruyak et al., 2016).

Accurately modelling the maternal behavioural profiles of PAAD, PPD or GDM in rodents is difficult; therefore, looking at measures of maternal care behaviour with diet manipulation in a rodent model is more feasible. Maternal behaviours in rodents are highly stereotyped behaviours triggered by estrogen and oxytocin changes at parturition (Rosenblatt, 1994). The first avenue of investigation is to determine if probiotics can shift

maternal care behaviour tendencies in a rodent model. I have previously found that acute treatment with the probiotic Lacidofil® (*Lacticaseibacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052) did increase active (i.e., licking/grooming and arched-back nursing) maternal care behaviours in Long-Evans rats (O'Leary, 2019). There is conflicting evidence regarding whether a Western diet negatively impacts maternal care. Some studies have found decreased licking/grooming behaviours when dams are given a Western diet (45% kcal from fat, Research Diets D12451; Connor et al., 2012), whereas others have found increased licking/grooming levels in dams provided a Western diet (45% kcal from fat, Research Diets D12451; Kougias et al., 2018; 60% kcal from fat; Research Diets D12492; Purcell et al., 2011). The theories regarding why diet impacts maternal care include Western diets being more satiating, resulting in the dam engaging in a high frequency of maternal behaviour (Kougias et al., 2018), or dietary impacts on maternal hormones (Connor et al., 2012).

2.1.5 Probiotics, Stress, Metabolic Dysfunction and Inflammation

Due to the comorbidities between GDM and PPD and the association with Western diet consumption, it is essential to investigate how probiotics may impact molecular markers of stress, metabolic dysfunction, and inflammation during the perinatal period.

A diet's composition is, as previously mentioned, linked to gut microbiome composition, with factors such as fat, sugar and fibre content all influencing its profile and, potentially, its function (Barrett et al., 2018; Gomez-Arango et al., 2018; Ley et al., 2006). There is evidence that both pregnancy and a Western diet can cause shifts in the gut microbiome's composition, with the latter exacerbating the former (Pérez-Pérez et al.,

2020). Therefore, probiotic supplementation during pregnancy should be investigated for its ability to counteract some of the microbiome shifts and the effects on hormones such as leptin (Pérez-Pérez et al., 2020). Presently, some research demonstrates probiotics' ability to lower leptin and inflammation levels in a diet-induced obesity model (Al-muzafar & Amin, 2017).

Chronic inflammation can occur when there is repeated or continuous oxidative stress or a disproportionate intake of certain nutrients (Bilal et al., 2022). Further, metabolic disorders can cause chronic inflammation, also known as metabolic inflammation (Bilal et al., 2022). A Western diet can also result in a chronic, low-grade inflammatory state in the gut (García-Montero et al., 2021). This state is due to nutrient excess or nutrient deficiencies that subsequently affect the composition of the gut microbiome, which in turn affects the immune response (Bilal et al., 2022). To combat this kind of inflammation, probiotics such as *Lactobacillus* and *Bifidobacterium* strains may be used for their abilities to up-regulate anti-inflammatory cytokines (Campeotto et al., 2011; Konieczna et al., 2012) or their abilities to down-regulate pro-inflammatory cytokine activity (Foster et al., 2011).

Probiotic strains have different abilities regarding how they may help with chronic inflammation (Bilal et al., 2022). For example, *L. rhamnosus* R0011 bacteria assist with gut barrier maintenance and can down-regulate pro-inflammatory markers (Foster et al., 2011). *L. helveticus* R0052 bacteria can down-regulate pro-inflammation pathways and inhibit the adhesion of harmful bacteria (e.g., *Escherichia coli*, *Salmonella*) within the gut (Foster et al., 2011). In addition to aiding with inflammation, probiotics may also help with the anxiety and depressive symptoms associated with PPD. For example, in a rat model of PPD, it was found that *Lactobacillus casei* treatment reduced behavioural anxiety- and

depressive-like symptoms (obtained from the sucrose preference test, tail suspension test, elevated plus maze and forced swim tests) when compared to untreated control rats (Y. Yang et al., 2022). The administration of this probiotic also reduced malondialdehyde (MDA) levels, a marker of oxidative stress in the treated rats (Y. Yang et al., 2022). Furthermore, research demonstrates the positive correlations between maternal brain dopamine and levels of Bifidobacteria and Lactobacilli in the gut (Krishna et al., 2015). However, the full extent of the microbiota's effects on stress reactivity and anxiety is not known, although it is clear from existing research that microbes are crucial in the HPA axis response (Sudo et al., 2004). Furthermore, probiotic treatment with *Lactobacillus* strains has shown promising abilities to reduce stress hormone levels. The strain *Lactobacillus farciminis* reduced stress-related increases in ACTH and corticosterone in a rat model of acute psychological stress (Ait-Belgnaoui et al., 2012). *Lactobacillus rhamnosus* JB-1 treatment reduced corticosterone levels in mice exposed to the forced swim test, which evaluates coping strategies to acute inescapable stress (Bravo et al., 2011).

Many studies investigating probiotic administration during human pregnancy begin the intervention later (around 32 to 35 weeks' gestation). However, this late administration means that the probiotics may be given too late to influence the mother's health status and specific developmental processes in the offspring that occur earlier. The maternal microbiome changes across trimesters, such that in the third trimester, there are greater abundances of the phyla Proteobacteria and Actinobacteria, as demonstrated in germ-free mice inoculated with either first or third trimester human gut microbes (Koren et al., 2012). Additionally, the third trimester has greater low-grade inflammation compared to the first-trimester mice, as indicated by increases in cecal cytokine levels (Koren et al., 2012).

Furthermore, studies investigating probiotics and food allergies in children found that exposure of the mother to pollen during the first trimester of pregnancy was sufficient to increase allergy risk in the children (Pyrhönen et al., 2012). Therefore, for the present study, I investigated maternal diet and probiotic administration beginning six weeks before conception to maximize the likelihood of detecting an effect of the probiotic on maternal health outcomes.

2.1.6 Objectives and Hypotheses

My first objective was to observe maternal care behaviours in Long-Evans rats during the first postnatal week and investigate the effects of chronic diet and probiotic administration on maternal care expression. I hypothesized that Western diet (WD)-fed, probiotic-treated dams would have increased active maternal care levels (i.e., increased frequencies of licking/grooming and arched-back nursing behaviours) compared to their WD-fed, placebo-treated counterparts.

My second objective was to measure inflammation levels via protein markers (e.g., cytokines) in the dams' plasma since a WD is known to increase global inflammation. In contrast, probiotics may help modulate the inflammation response to diet. Therefore, I hypothesized that WD-fed, probiotic-treated dams would have lower plasma inflammatory markers than their WD-fed, placebo-treated counterparts.

My third objective was to measure metabolic marker levels in the dams' plasma to investigate if the WD disrupted metabolic function. I did not have a hypothesis about how probiotic administration may affect metabolic markers in the dams, as the probiotic Lacidofil® is not typically used in nutrition research. I did hypothesize that the WD-fed

dams would demonstrate disrupted metabolic marker patterns (e.g., increased leptin and decreased ghrelin levels).

Our fourth objective was to measure the dams' total plasma corticosterone (bound and unbound) as an indicator of stress. I hypothesized that WD administration would increase dams' plasma corticosterone but that probiotic administration would help to mitigate the effects of the Western diet on corticosterone levels, such that WD-fed, probiotic-treated dams would have lower plasma corticosterone than their WD-fed, placebo counterparts. I also hypothesized that corticosterone levels of WD-fed, placebo dams would positively correlate with their pro-inflammatory marker levels. In contrast, this correlation would not be present in the Western diet-fed, probiotic-treated dams.

METHODS

2.1.7 Animals, Breeding and Probiotic Administration

This experiment was conducted under the animal ethics protocol #20-136. The experimental procedures performed throughout this experiment were done per the guidelines of the Canadian Council on Animal Care (CCAC). They were approved by the Dalhousie University Committee on Laboratory Animals (UCLA).

Sixty Long-Evans hooded rats (specific pathogen-free, viral antibody free), 20 males (225-250 g), 20 females (200-225 g) and 20 females (175-200 g) were purchased (Charles River Laboratories, Raleigh, NC, USA). The female rats were split into two separate cohorts, such that the 20 females that weighed 175-200 g upon arrival were treated the same as the heavier females, but their timeline was two weeks later. The males

purchased were used in another experiment first to gain breeding experience but were not subject to any experimental treatments themselves (e.g., pharmacological, probiotic, diet).

Upon arrival, the animals were quarantined for two weeks before the experiment commenced as per facility protocols. Post-quarantine, rats were randomly assigned to either the "placebo" or the "probiotic" colony rooms, which were identical in size and layout. This configuration resulted in 20 female and 10 male rats per room. The male rats received no placebo or probiotic treatment during this experiment. During quarantine, all rats received food (Laboratory Rodent Diet 5001, LabDiet®, St. Louis, MO, USA) and tap water *ad libitum.*

Both colony rooms had a 12h:12h reversed light-dark cycle (lights off at 1000h). The temperature of the colony room was maintained at 20°C ± 2°C. All animals were housed in same-sex pairs in polypropylene cages (47 cm x 24 cm x 20.5 cm) with wire lids containing animal bedding (Fresh Bed, Shaw Resources, Shubenacadie, NS, Canada) and a black polyvinyl chloride (PVC) tube for enrichment (12 cm length, 9 cm diameter). After quarantine, the females were randomly assigned a diet and treatment condition. Both diet and placebo (or probiotic) administration began on the day immediately following the end of quarantine (this was considered "Gestational Day [GD] -42"). Beginning from GD -42, females were weighed weekly. The breakdown of the number of animals per experimental group is shown in Table 2.1, and the experimental timeline is illustrated in Figure 2.1.

Table 2.1 Experimental group sample sizes.

Treatment	Diet	Starting *n*	Final *n*
Placebo	Standard	10	8
	Western	10	7
Probiotic	Standard	10	8
	Western	10	8

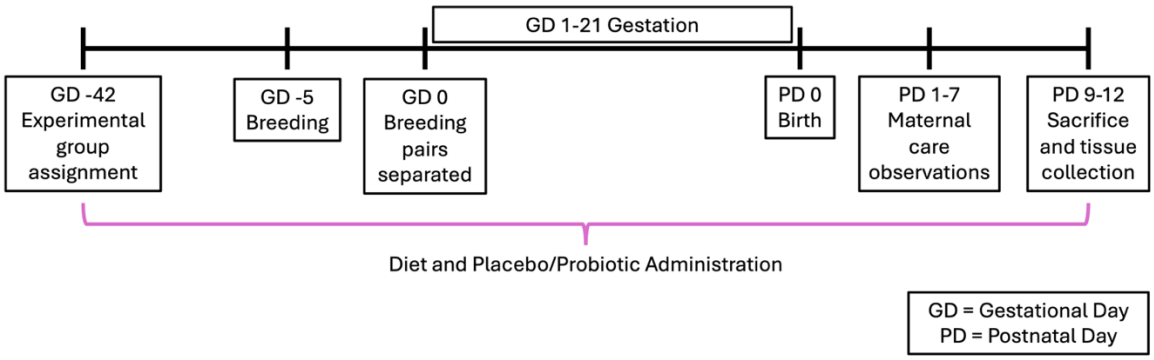

Figure 2.1 An experimental timeline illustrating the study.

The Western diet (Cat. #D12079B, Research Diets, New Brunswick, New Brunswick, NJ, USA) and the standard diet (the same standard diet provided during quarantine) were both provided *ad libitum* (see Table 2.2 for the diet macronutrients, and Appendix A for the detailed diet information sheets).

Table 2.2 Macronutrient formulations for the Standard and Western diets.

Diet	% Fat	% Carbohydrates	% Protein	Kcal/g
Standard	13	58	29	3.4
Western	40	43	17	4.7

Placebo or probiotic administration was done via oral syringe, using a modified version (Myles, O'Leary, Romkey, et al., 2020) of a published protocol (Tillmann & Wegener, 2018). To ensure that there was no cross-contamination between the "placebo" and "probiotic" rooms, according to a previously described protocol (Myles, O'Leary, Romkey, et al., 2020). The probiotic treatment used in this study was Lacidofil® (*Lacticaseibacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052; Foster et al., 2011). Of note, *Lacticaseibacillus rhamnosus* R0011 was updated from its former designation of "*Lactobacillus rhamnosus*." The placebo and probiotic solutions were prepared daily from lyophilized powder (see Table 2.3 for the placebo and probiotic vehicle formulation) and stored at 4°C (± 4 °C). The probiotic solution containing the *Lacticaseibacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 strains was prepared at a dosage of 500 million (10^8) colony-forming units (CFU) per 0.5 mL of reverse-osmosis water daily. The administration time was consistent, occurring between 1000 – 1100h. Animals were monitored daily by both animal care staff and experimenters for general wellbeing. Although it was not explicitly tracked, no sickness behaviour was noted for any of the animals receiving the placebo or the probiotic.

Table 2.3 Placebo and probiotic (Lacidofil®) vehicle formulation.

Ingredient	% of Formulation	Amount in Grams
Maltodextrin	85	425
Magnesium Stearate	3	15
Milk Powder	11.6	58
Ascorbic Acid	0.4	2

On GD -5, females were paired with experienced male breeders for five consecutive days. The females continued to be fed their respective diets and placebo or probiotics during this period. After separating females from the breeder males, they were pair-housed with the same female they had lived with before breeding. On approximately GD 12-16 (this window is approximate because of the 5-day breeding protocol; I cannot truly pinpoint when gestation started for each female), I single-housed all female rats. They were given Enviro-dri® (Shepherd Speciality Papers©, Watertown, TN, USA) nesting material.

Beginning on GD 15, I began checking for litters twice daily (once in the morning during the placebo/probiotic administration (1100h) and once in the afternoon (between 1500h and 1700h). If a litter was found during these checks, this was considered Postnatal Day (PD) 0 and all pups were sexed and weighed. The dam was also weighed and she and her litter were given a fresh cage at this time.

2.1.8 Maternal Care Observations

Starting on PD 1 (the day after birth), dams and their litters were observed for their maternal care behaviours for seven consecutive days (until PD 7). Cage changes were only done during the maternal care observation period if the cage was soiled to minimize disruption to the dam and her offspring. A trained observer scored the observations manually in real-time for 60 minutes at five time points throughout the day: 0830h, 1400h, 1700h, 2000h, and 2300h (see Appendix B for the observation sheet used). For all observation periods, the frequency of maternal care behaviours was recorded every three minutes (Korgan et al., 2016). The maternal care behaviours scored included passive nursing, blanket posture nursing (also known as arched-back nursing level “1”), arched-

back nursing levels “2” through “4”, licking and grooming pups, separated pups, pup retrieval, pup move, no contact pups, self-feeding, nest building, self-grooming, and pup contact (see Appendix C for a maternal care behaviour ethogram; adapted from Champagne et al., 2003). The dams and their offspring were sacrificed between PD 9 and 12. Dams were separated from their litters for two hours before being sacrificed so that breast milk could be collected.

2.1.9 Sacrifice and Tissue Collection

The offspring were sacrificed within one hour of being separated from the dam. All rats were sacrificed with Euthanyl (Pentobarbital Sodium; DIN 00141704, Bimeda MTC Animal Health Inc., Cambridge, ON, Canada), and surgical plane anesthetization was confirmed by checking that the toe pinch reflex was absent. Dams were decapitated via guillotine; offspring were decapitated via sharp surgical scissors.

2.1.10 Plasma Collection and Preparation

Whole trunk blood was collected in a microtube containing 15 µL of sodium heparin (DIN 02303086, Sandoz Canada Inc., Boucherville, QC, Canada) and placed on ice immediately until plasma could be processed later that day. All plasma samples were prepared by first centrifuging the whole blood at 4°C for 15 minutes at 1,000 × *g*. The supernatant was collected and pipetted into a new microtube, followed by a second centrifugation at 4°C for 10 minutes at 10,000 × *g*. The plasma from the second centrifugation was pipetted into a new microtube for long-term storage at -80°C.

2.1.11 Organ Collection and Preservation

Fresh, whole brains were collected from offspring and dams and flash-frozen in chilled isopentane (approximately -70°C). Spleen and caecum were also dissected from the dams and flash-frozen on dry ice. All tissue samples were stored at -80°C to await processing.

2.1.12 Immunoassay Protocols

2.2.6.1 Plasma Corticosterone Enzyme Immunoassay (ELISA)

Assay-Specific Plasma Preparation. Before beginning the assay, the Dissociation Reagent included in the DetectX® Corticosterone Enzyme Immunoassay Kit (Cat. #K014-H1, Arbor Assays, Ann Arbor, MI, USA) was brought to room temperature, and 5 µL was added to new microtubes. Then, 5 µL of each sample was added to a microtube containing the Dissociation Reagent and vortexed for approximately 10 seconds to mix. The samples were then incubated at room temperature for five minutes before diluting with 490 µL of Assay Buffer, resulting in a 1:100 dilution.

Enzyme Immunoassay. First, all kit reagents and samples were brought to room temperature for 30 minutes before use. The assay was prepared according to the manufacturer's instructions for the 50 µL assay format, which has a concentration range of 39.063 pg/mL to 10,000 pg/mL. Briefly, 50 µL of the samples and standards were added to the plate in duplicate. Then, 75 µL of Assay Buffer was added to the non-specific binding (NSB) wells, and 50 µL of Assay Buffer was added to the maximum binding (B0) wells. A multichannel pipette was used to add 25 µL of Corticosterone Conjugate to all wells. This step was followed by adding 25 µL of the Corticosterone Antibody to all wells except

for the NSB wells. The plate was covered using a plate sealer and incubated for one hour at room temperature (21°C) on a plate shaker (moving in a horizontal circular motion) set to 500 RPM (Note. The protocol said to shake at 700-900 RPM; however, 500 RPM was the maximum speed for the plate shaker). After incubation, the plate was aspirated and washed four times using 300 µL of prepared Wash Buffer per well. After the washes, the plate was tapped dry on layered paper towels covered by a large KimWipe (Cat. # CA10805-905, VWR Canada) to prevent debris from the paper towel from going into the plate wells. Next, using a multichannel pipette, 100 µL of the TMB Substrate was added to all wells, followed by a 30-minute incubation at room temperature with no shaking. At this step, the plate was also incubated in the dark. After this last incubation, 50 µL of Stop Solution was added to all wells and the plate was subsequently read on a plate reader (Multiskan FC, ThermoFisher Scientific) at 450 nm. The results were obtained using the SkanIt™ software (Research Edition, Version 6.0.2, ThermoFisher Scientific) and exported into Microsoft Excel (Microsoft Excel for Mac, Version 16.84).

2.2.6.2 Multiplex Analysis of Inflammation Markers

Assay-Specific Plasma Preparation. For this assay, I first diluted all plasma samples to a 1:4 dilution using the Sample Diluent from the Bio-Plex Pro™ Rat Cytokine 23-Plex Assay (Cat. #12005641, Bio-Rad).

Multiplex Assay. The Luminex® bead-based assay, Bio-Plex Pro™ Rat Cytokine 23-Plex Assay (Cat. #12005641, Bio-Rad), was used to detect inflammation analytes in the dams' plasma (see Appendix D for a list of inflammation markers and their functions; adapted from the Rat Genome Database; Shimoyama et al., 2015). The assay was prepared according to the manufacturer's instructions. First, the BioPlex® 200 system (Cat.

#171000201, Bio-Rad) was calibrated per the manufacturer's instructions using the calibration kit (Cat. #171203060, Bio-Rad). Briefly, plasma samples were thawed on ice and diluted in a 1:4 ratio using the provided sample diluent. The vial containing a lyophilized standard was reconstituted using 500 µL of sample diluent and incubated for 30 minutes before preparing the standard curve in a dilution series. The antibody-coupled magnetic beads were vortexed, diluted using assay diluent, and vortexed again and 50 µL of the bead solution was added to each well on the provided 96-well plate. The plate was then attached to a handheld magnetic washer (Cat. #171020100, Bio-Rad), and the plate was washed twice with 100 µL of wash buffer added to each well. Afterwards, 50 µL of samples, standards and blanks were added to the appropriate wells. The plate was then covered with sealing tape and incubated on a plate shaker at 850 RPM at room temperature for 1 hour. The plate was washed three times, ensuring that the plate was back on the handheld magnet when decanting the wash buffer. Then, 25 µL detection antibodies were added to each well, and the plate was incubated on the plate shaker at 850 RPM at room temperature for 30 minutes. After the incubation, the plate was rewashed three times, and 50 µL Streptavidin-Phycoerythrin was added to each well. The plate was again incubated at 850 RPM at room temperature for 10 minutes. The plate was washed three times; then, the magnetic beads were resuspended in assay diluent, put on the plate shaker at 850 RPM for 30 seconds, and read in the BioPlex® 200 System. The plate was read using the "High PMT, RP1" settings in the assay kit's protocol.

2.2.6.3 Multiplex Analysis of Diabetes Markers

Assay-Specific Plasma Preparation. Before assaying the dams' plasma samples with the Bio-Rad Rat Diabetes Assay, protease inhibitors had to be added. I dissolved 7.4

mg of DPP-IV inhibitor (Cat. #K4264, Sigma Aldrich) into 2 mL sodium chloride (NaCl, Cat. #S8776-100ML, Sigma Aldrich) and 26.67 mg of aprotinin (Cat. #A3428, Sigma Aldrich) into 2 mL NaCl. Plasma samples were aliquoted into 100 µL, 10 µL of the 10 mM DPP-IV inhibitor solution was added to each sample, followed by 10 µL of the 1.3% aprotinin solution.

Multiplex Assay. Before beginning the assay, the BioPlex® System was calibrated and left to warm up before use. All assay reagents were removed from the fridge and left to come to room temperature (RT). Once reagents reached RT, the protease inhibitor-treated samples were diluted at a 1 in 4 dilution, using sample diluent.

For the assay preparation, I first reconstituted the standards. Once reconstituted, the standard was incubated on ice for 30 minutes. After incubating, the standard curve was created as instructed, using a fourfold dilution series.

Next, the 20X coupled beads were vortexed and diluted to 1X in Bio-Plex Assay Buffer. For this, each molecular marker had its vial of 20X beads and 288 µL of beads from each marker was added to a conical centrifuge tube. The total bead volume (1,440 µL) was subtracted from the total volume needed for a 1X dilution, and 4,320 µL of Assay Buffer was added to achieve the correct dilution. The resulting 1X mixed beads were then vortexed again, and 50 µL was added to each well. Then, after attaching the handheld magnetic washer, the plate was washed twice with 100 µL of Bio-Plex Wash Buffer.

The standards, samples and blanks were added (50 µL per well). The plate was then sealed with a foil plate seal and incubated at room temperature on a plate shaker (850 RPM) for 1 hour. Ten minutes before the incubation step finished, I diluted the 20X detection

antibodies to 1X as directed. In brief, 150 µL of each 20X antibody was added to 2,250 µL of Detection Antibody Diluent to achieve a final 1X antibody solution.

Post-incubation, the plate was washed three times (attached to the plate magnet) with 100 µL Bio-Plex wash buffer. The 1X detection antibodies were vortexed, and 25 µL was added to each well.

The plate was again incubated at RT for 30 minutes at 850 RPM. Before the end of the incubation period, the 100X streptavidin-phycoerythrin (SA-PE) was diluted to 1X as instructed. The plate was secured to the magnet and then washed three times with 100 µL wash buffer, and then 50 µL of 1X SA-PE was added to each well. The plate was again incubated at room temperature for 10 minutes on a plate shaker (850 RPM).

After this last incubation, the plate was placed on the plate magnet and washed three times with 100 µL wash buffer. Then, the beads were resuspended in 125 µL of assay buffer and shaken at 850 RPM for 30 seconds. The plate was then read using the Bio-Plex 200 System, with the "High PMT, RP1" setting listed in the assay kit's protocol.

The plate data was obtained using the Bio-Plex Manager software (Version 6.0) and exported to a Microsoft Excel sheet (Microsoft Excel for Mac, Version 16.84).

2.1.13 Statistical Analyses

All statistical analyses were performed using Jamovi (Version 2.3.21.0; Love et al., 2022). All measures were analyzed using two-way factorial ANOVAs, with maternal diet (Standard versus Western) and maternal treatment (placebo versus probiotic) as the factors. Specifics regarding the handling of outliers are detailed in the appropriate results section.

The correlations between maternal plasma corticosterone and inflammatory markers were completed using Pearson correlations.

RESULTS

2.1.14 Weekly Dam Weights

The rats were weighed on the day they started their respective diet and treatment, at which time I observed no significant differences in their weights. Interestingly, at the end of the experiment, on the sacrifice day, I also observed no significant differences in the rats' weights. The only significant weight difference I found was at six weeks into the diet and treatment administration, the WD-fed dams weighed significantly more (M = 297.00; SD = 33.20, $F_{(1, 27)} = 14.14$, $p < .001$, $\eta^2 = .342$) compared to their SD-fed counterparts (M = 262.00; SD = 12.90).

2.1.15 Maternal Care

For the maternal care behaviour measures, I found statistically significant results for the following behaviours: licking/grooming (LG), arched-back nursing level "2" (ABN 2), water intake (W), food intake (F), self-grooming (SG) and active care (LG and ABN 2-4). I observed that WD-fed dams engaged in significantly more LG bouts (M = 105.00; SD = 33.40, $F_{(1, 27)} = 6.94$, $p = .014$, $\eta^2 = .190$) compared to SD dams (M = 79.30; SD = 19.10; see Figure 2.2.A). Conversely, I found that probiotic-treated dams engaged in more ABN 2 (M = 237.00; SD = 37.90, $F_{(1, 27)} = 4.85$, $p = .036$, $\eta^2 = .133$) compared to their placebo-treated counterparts (M =205.00; SD = 45.70; see Figure 2.2.B). SD-fed dams engaged in a greater frequency of W (M = 22.60; SD = 8.28; $F_{(1, 27)} = 48.57$, $p < .001$, $\eta^2 = .620$)

compared to WD-fed dams (M = 6.73; SD = 3.20). Similarly, SD-fed dams also engaged in more F bouts (M = 107.00; SD = 19.70; $F_{(1, 27)} = 117.24$, $p < .001$, $\eta^2 = .811$) compared to WD-fed dams (M = 39.80; SD = 12.90). WD-fed dams also performed more SG behaviours (M = 83.50; SD = 18.80, $F_{(1, 27)} = 121.06$, $p < .001$, $\eta^2 = .403$) compared to SD-fed dams (M = 55.60; SD = 16.80). I found that WD-fed dams engaged in more LGABN 2/3 behaviour, which represents when dams were concurrently performing LG and ABN level 2 or 3, (M = 91.7, SD = 32.3; $F_{(1, 27)} = 5.04$, $p = .033$, $\eta^2 = .146$) than SD-fed dams (M = 70.8; SD = 16.4; see Figure 2.2.C). Lastly, as an exploratory measure, I found that the probiotic-treated dams engaged in more "active care" behaviours, comprising LG and ABN 2-4 (M = 402.00; SD = 76.90; $F_{(1, 27)} = 4.58$, $p = .041$, $\eta^2 = .132$), than their placebo-treated counterparts (M = 344.00; SD = 76.80; see Figure 2.2.D).

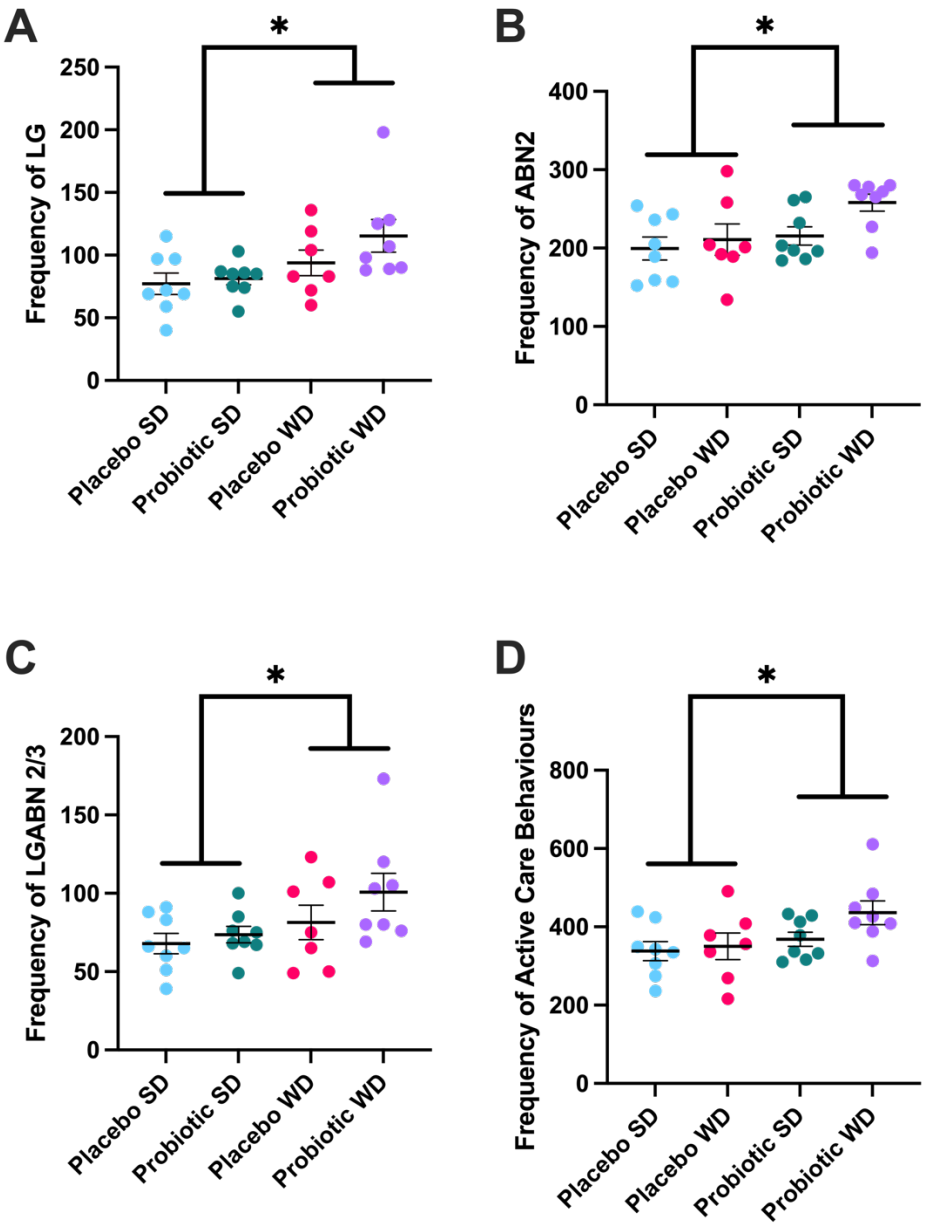

Figure 2.2 A) The total frequency of licking/grooming (LG) behaviours across postnatal days 1-7 (mean, SD), $p < .05$. B) The total frequency of arched-back nursing "2" (ABN 2) behaviours across postnatal days 1-7 (mean, SD), $p < .05$. C) The total frequency of the co-occurrence of LG and ABN 2/3 behaviours across postnatal days 1-7 (mean, SD), $p < .05$. D) The total frequency of all "active care" maternal behaviours, including LG and ABN 2-4 across postnatal days 1-7 (mean, SD), $p < .05$.

2.1.16 Maternal Inflammation Markers

There were 23 analytes in the Bio-Plex Pro™ Rat Cytokine 23-Plex Assay, and only thirteen were measurable in the present experiment. As such, statistics were only performed on the analytes that were measured. Outliers were calculated as three times the standard deviation, and samples were excluded if they exceeded this value. Samples were excluded if the coefficient of variation was greater than 20% or if more than 50% of the samples for a given analyte did not read. The analytes that were not analyzed included: IFN-γ, IL-2, IL-4, IL-6, IL-12p70, IL-13, IL-17, M-CSF, TNF-α and VEGF.

I found no significant effect of maternal diet or treatment or their interaction on plasma levels of G-CSF, IL-1α, IL-5, IL-10, IL-18, MIP-3α, or RANTES. However, I did find several significant main effects of diet on some of the analytes, where Western diet-fed dams had significantly higher levels of the analyte than standard diet-fed dams, regardless of the analyte's role (e.g., pro- or anti-inflammatory, regulatory).

There was a significant effect of maternal diet on plasma GM-CSF levels, where Western diet-fed dams had higher GM-CSF levels (M = 767.00; SD = 1063.00, $F_{(1, 24)}$ = 7.09, p = .014, η^2 = .189), compared to standard diet-fed dams (M = 116.00; SD = 128.00; see Figure 2.3A).

There was a significant effect of maternal diet on plasma IL-1β levels, where Western diet-fed dams had higher IL-1β levels (M = 791.00; SD = 1,111.00, $F_{(1, 25)}$ = 6.85, p = .015, η^2 = .186), compared to standard diet-fed dams (M = 124.00; SD = 125.00; see Figure 2.3B).

There was a significant effect of maternal diet on plasma MIP-1α levels, where Western diet-fed dams had higher levels (M = 163.00; SD = 260.00, $F_{(1, 25)}$ = 5.20, p = .031, η^2 = .140), compared to standard diet-fed dams (M = 33.00; SD = 36.50; see Figure 2.3C).

There was a significant effect of maternal diet on plasma GRO/KC levels, where Western diet-fed dams had higher GRO/KC levels (M = 259.00; SD = 138.00, $F_{(1, 25)}$ = 10.30, p = .004, η^2 = .283), compared to standard diet-fed dams (M = 121.00; SD = 99.30; see Figure 2.3D).

There was a significant effect of maternal diet on plasma IL-7 levels, where Western diet-fed dams had higher IL-7 levels (M = 1,042.00; SD = 1,667.00, $F_{(1, 23)}$ = 5.03, p = .035, η^2 = .154), compared to standard diet-fed dams (M = 116.00; SD = 125.00; see Figure 2.3E).

There was a significant effect of maternal diet on plasma MCP-1 levels, where Western diet-fed dams had higher MCP-1 levels (M = 2,508.00; SD = 2,888.00, $F_{(1, 26)}$ = 6.67, p = .016, η^2 = .177), compared to standard diet-fed dams (M = 845.00; SD = 542.00; see Figure 2.3F).

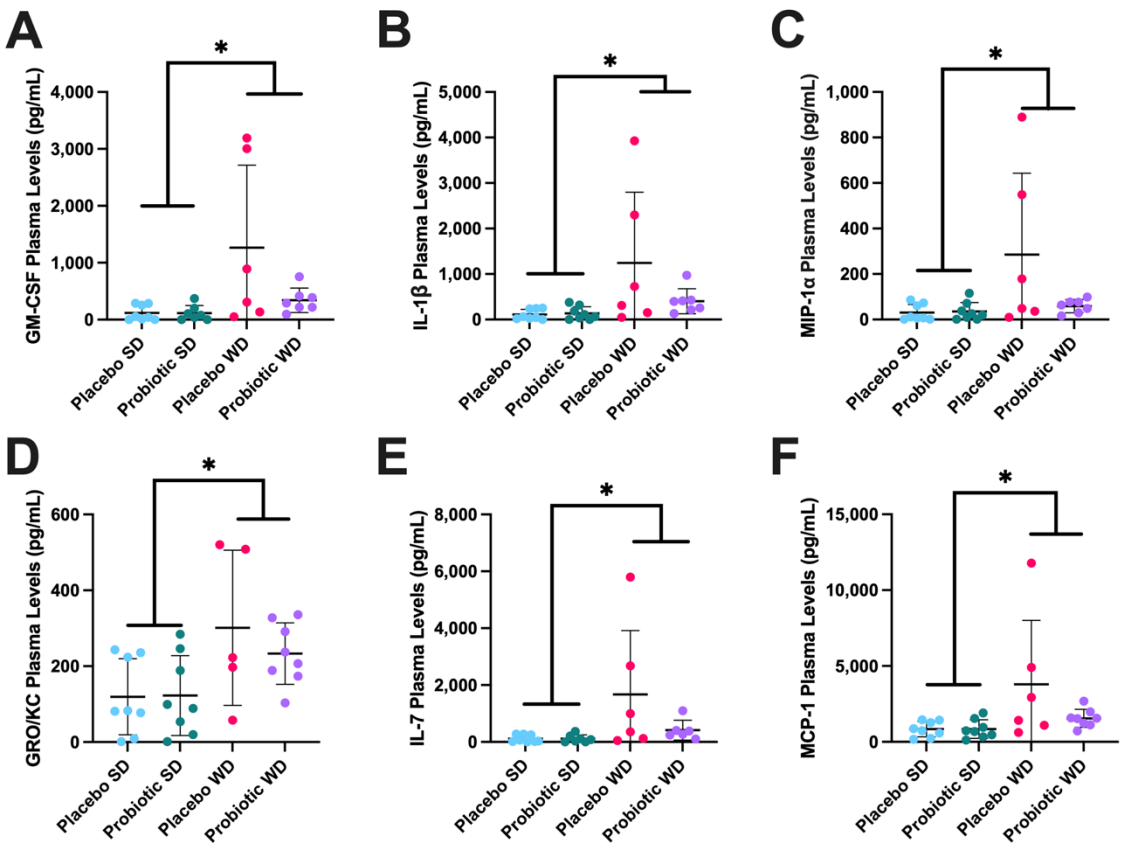

Figure 2.3 A) Maternal plasma granulocyte-macrophage colony-stimulating factor (GM-CSF; mean, SD) concentrations in pg/mL, $p < .05$. B) Maternal plasma interleukin (IL)-1 beta (IL-1β; mean, SD) concentrations in pg/mL, $p < .05$. C) Maternal plasma macrophage inflammatory protein-1 alpha (MIP-1α; mean, SD) concentrations in pg/mL, $p < .05$. D) Maternal plasma growth-regulated oncogenes/keratinocyte chemoattractant (GRO/KC; mean, SD) concentrations in pg/mL, $p < .05$. E) Maternal plasma interleukin (IL)-7 (mean, SD) concentrations in pg/mL, $p < .05$. F) Maternal plasma monocyte chemoattractant protein 1 (MCP-1; mean, SD) concentrations in pg/mL, $p < .05$.

2.1.17 Maternal Diabetes Markers

The Diabetes 5-Plex assay measured glucagon, leptin, ghrelin, GLP-1 and PAI-1; however, only glucagon, leptin and ghrelin were quantifiable. As such, I only performed statistics on these successfully measured analytes. Outliers were calculated as three times

the standard deviation, and samples were excluded if they exceeded this value. Samples with a coefficient of variation exceeding 20% were also excluded.

No significant effect of maternal diet or treatment was found for plasma glucagon levels. There was a significant effect of maternal diet on plasma leptin levels, where Western diet-fed dams had higher leptin levels (M = 2,075.00; SD =1,671.00, $F_{(1, 26)}$ = 11.20, p = .002, η^2 = .298), compared to standard diet-fed dams (M = 585.00; SD = 193.00; see Figure 2.4A. There was also a significant effect of maternal diet on plasma ghrelin levels, where Western diet-fed dams had lower plasma ghrelin levels (M = 740.00; SD = 367.00, $F_{(1, 26)}$ = 21.41, p < .001, η^2 = .435), compared to standard diet-fed dams (M = 1,445.00; SD = 455.00; see Figure 2.4B.

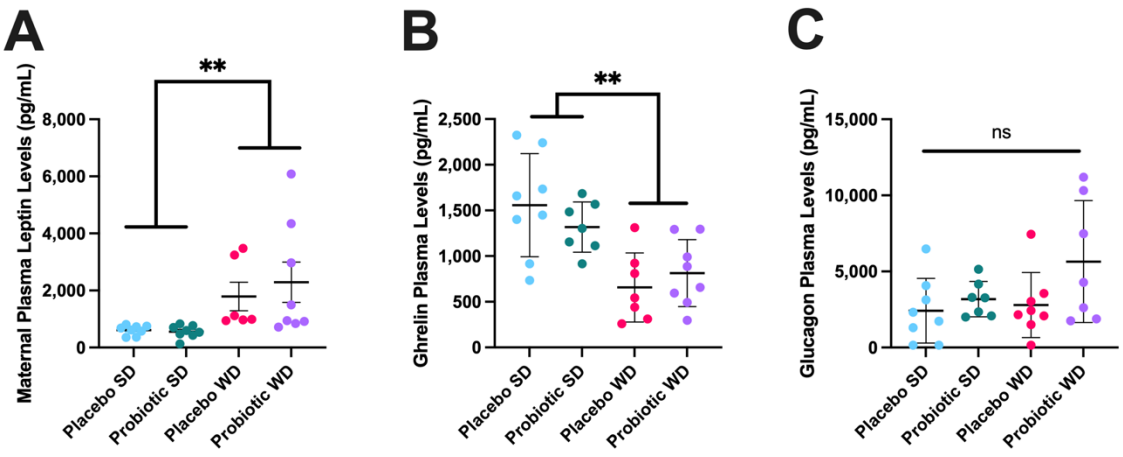

Figure 2.4 A) Maternal plasma leptin concentrations in pg/mL (mean, SD), p < .01. B) Maternal plasma ghrelin concentrations in pg/mL (mean, SD), p < .01. C) Maternal plasma glucagon concentrations are in pg/mL (mean, SD), with no significant differences.

2.1.18 Maternal Plasma Corticosterone

A significant main effect of maternal diet on plasma corticosterone levels was detected, where Western diet-fed dams had significantly higher plasma corticosterone (M

= 580,171.00; *SD* = 141,764.00, $F_{(1, 26)} = 18.52$, $p < .001$, $\eta^2 = .414$) than standard diet-fed dams (*M* = 303,521.00; *SD* = 191,223.00; see Figure 2.5). One WD animal was excluded from analyses because their sample had a correlation coefficient greater than 20%.

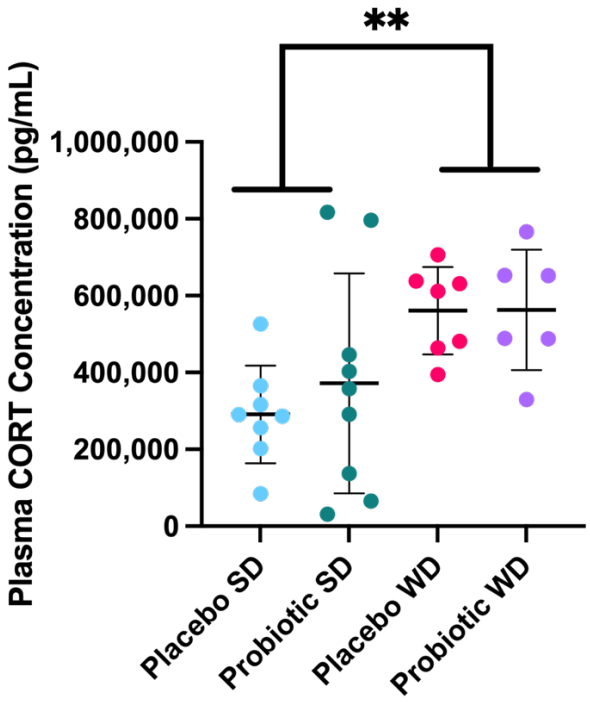

Figure 2.5 Maternal plasma corticosterone (CORT; mean, SD) concentrations in pg/mL, $p < .05$.

2.1.19 Maternal Corticosterone and Inflammatory Marker Correlations

In this experiment, I found several significant correlations between plasma corticosterone and the measurable inflammatory marker levels across all dams (see Table 2.4). More specifically, only a few significant correlations were found in placebo-treated, probiotic-treated or standard diet-fed dams. Interestingly, there were no significant correlations unique to the Western diet-fed dams. IL-1α levels were negatively correlated with corticosterone levels in the placebo-treated dams, whereas IL-1β levels were

positively correlated with corticosterone in the placebo-treated dams. GRO/KC levels were positively correlated with corticosterone levels in the probiotic-treated dams. Lastly, MIP-3α levels were negatively correlated with corticosterone in the standard diet-fed dams.

Table 2.4 Pearson correlations between maternal plasma corticosterone levels (pg/mL) and maternal plasma inflammatory marker levels (pg/mL).

Inflammatory Marker	**Corticosterone[a]**				
	Placebo	Probiotic	Standard Diet	Western Diet	Total
GM-CSF	0.624*	-	-	-	0.423*
IL-1α	-0.605*	-	-	-	-
IL-1β	0.563*	-	-	-	0.386*
MCP-1	0.602*	-	-	-	0.398*
MIP-1α	0.603*	0.615*	-	-	0.382*
MIP-3α	-	-	-0.653**	-	-
GRO/KC	-	0.588*	-	-	0.488**

*$p < .05$
**$p < .01$
[a]One dam from the probiotic-treated, Western diet grouping was not included in the correlations because the corticosterone measurement was not viable.

DISCUSSION

Six weeks into the diet and probiotic administration, I found that the WD-fed dams weighed significantly more than the SD-fed dams. This weight difference was no longer significant when the dams were sacrificed in the second week postpartum (eleven weeks into the diet and probiotic administration). When observing maternal care, I found the WD-fed dams performed significantly more LG behaviour than their SD-fed counterparts and the probiotic-treated dams engaged in more ABN 2 than their placebo-treated counterparts. Overall, the maternal care observations showed no interaction between treatment and diet. In the inflammatory marker data, I saw a trend of the WD-fed dams having elevated levels of inflammatory markers, regardless of the inflammatory marker's role (i.e., pro-

inflammatory versus anti-inflammatory). The metabolic markers, leptin and ghrelin, were also affected by maternal diet status, such that WD-fed dams had increased leptin levels and decreased ghrelin levels. Lastly, maternal corticosterone levels were also impacted by diet, where WD-fed dams had increased corticosterone levels compared to their SD-fed counterparts. Interestingly, there were only a few significant correlations between maternal plasma corticosterone and inflammatory marker levels.

2.1.20 The Impact of Western Diet and Pregnancy on Weight Gain

In the present study, I found that although WD female rats showed increased weight gain relative to their SD-fed counterparts at six weeks of diet administration (before breeding), this difference in weight disappeared in the postpartum period. My findings are like those of Frihauf and colleagues (2016), who found that in a rat model of diet-induced obesity (using a 32% kcal fat diet), the dams' pre-pregnancy weights were increased. In this study, the WD-fed dams did not differ from the control dams' weight gain across pregnancy (Frihauf et al., 2016).

These results are also consistent with work showing that estrogen may be protective against some outcomes associated with WD consumption, including weight gain (Buscato et al., 2021). E2 and E4 are natural estrogens, where E4 can regulate gene transcription, like E2 (the primary estrogen in human adults). A study performed by Buscato and colleagues (2021) investigated the potential of E4 to prevent metabolic dysfunction in ovariectomized female wild-type C57Bl/6 mice provided a 9-week WD (42% kcal fat; Buscato et al., 2021). A subset of the mice had E4 pellets implanted, and their blood estrogen levels were monitored to ensure their clinical relevance (Buscato et al., 2021).

Mice implanted with E4 demonstrated significantly more energy expenditure during the animals' active phase, which may be one way in which estrogen administration proves protective against WD outcomes (Buscato et al., 2021).

Previous research in chronic obesogenic diet studies focusing on female rats has found mixed results. In a 2019 study by Skinner and colleagues, female Sprague-Dawley rats were fed a WD (45% fat, 33% sucrose; 4.7 kcal/g) for eight weeks. Interestingly, although the WD-fed animals had increased gonadal adiposity compared to their control (standard purified rat diet) counterparts, they did not weigh significantly more (Skinner et al., 2019). I did not examine gonadal adiposity in the present study, but I did find weight differences between the female rats at the eight-week mark, where the WD-fed females weighed more than the SD-fed females. Another study using a 7-week diet administration protocol in both male and female Long-Evans rats found no differences in the weights of the female rats at the 7-week mark (Myles, O'Leary, Smith, et al., 2020). This study by Myles and colleagues utilized the same WD as the current study, but their control diet was a calorie-matched diet from Research Diets, Inc. rather than a standard rat chow (Myles, O'Leary, Smith, et al., 2020). Although both studies (Myles, O'Leary, Smith, et al., 2020; Skinner et al., 2019) found that female rats did not gain significant weight following a chronic obesogenic diet, it must be noted that neither of these studies utilized pregnant rats. Since the rats utilized in the present study did experience pregnancy, this is a potential factor in my finding that obesogenic diet did not alter female weight at the 11-week mark.

Conversely to my findings and those of Skinner and colleagues (2019), a study that provided female Wistar rats either a standard chow or a WD chow (#D12079B from Research Diets = 21% fat, 50% carbohydrates, 20% protein) for 17 weeks found that

female WD-fed rats weighed significantly more than their standard chow-fed counterparts (B. Kramer et al., 2018). However, these female rats were also not pregnant at any point in this study (B. Kramer et al., 2018). Hence, these findings differ from my results, even though it is a chronic administration using the same diet. In a study investigating E2 effects on female Wistar rats provided an HFD (60.7% energy from fat), researchers found that HFD-fed females implanted with E2 pellets did not gain significant amounts of weight, compared to their control counterparts (Yokota-Nakagi et al., 2020). These results, in combination with my own, suggest that the heightened estrogen levels experienced during and after pregnancy likely protect against significant weight gain.

2.1.21 Western Diet Increases Licking/Grooming Behaviour

My maternal care results show that dams fed the WD engaged in more LG behaviour, which is partly consistent with previous research. Like the findings of Kougias and colleagues (2018) and Purcell and colleagues (2011), I also found that WD-fed dams engaged in more LG behaviours than the control group. A study that split maternal care observation periods into their "light phase" versus "dark phase" found that WD-fed dams engaged in more LG behaviour, specifically during the "dark phase" of their cycle (Abuaish et al., 2018). These results (Abuaish et al., 2018; Kougias et al., 2018; Purcell et al., 2011) are inconsistent with work performed by Connor and colleagues (2012) that showed WD decreased LG behaviour.

The differences observed between these studies does not appear to be due to the length of diet administration, as both Kougias and colleagues (2018) and Connor and colleagues (2012) began diet administration on GD 0 (as determined by the presence of

sperm in the vaginal canal). The longest diet administration began three weeks before breeding (Abuaish et al., 2018) with the shorter beginning on GD 2 (Purcell et al., 2011). However, the results of Abuaish and colleagues versus Purcell and colleagues are similar.

Interestingly, the present study and two other studies on diet administration and maternal care utilized Long-Evans rats (Abuaish et al., 2018; Purcell et al., 2011). In contrast, Connor and colleagues (2012) utilized Wistar rats. Differences in maternal behaviour across different rat strains have been observed previously. In a study of maternal alcohol consumption, researchers found differences in how maternal care behaviours were impacted between Sprague-Dawley versus Long-Evans rats (Popoola et al., 2015). In this study, the Sprague-Dawley dams had more ABN occurrences overall than the Long-Evans dams (Popoola et al., 2015). Maternal alcohol consumption also differentially affected the two strains, such that ABN 1 was increased in Sprague-Dawley dams exposed to alcohol, whereas alcohol exposure decreased ABN 1 in Long-Evans dams (Popoola et al., 2015). Similarly, in a gestational unpredictable stress model, researchers found differences in the maternal care behaviours of Sabra rats, as compared to Sprague-Dawley rats (Poltyrev & Weinstock, 1999). In response to the repeating stress across gestation, Sabra rats had increased frequencies of LG and ABN behaviours compared to Sprague-Dawley dams (Poltyrev & Weinstock, 1999). These findings, in addition to the findings regarding diet administration in rat dams, demonstrate that the genetics of the rat strain interact with the experimental manipulation.

2.1.22 Probiotics Affect Maternal Care Without Interacting with Diet Administration

Previously, I found that acute Lacidofil® administration during pregnancy and lactation (a total of 42 days of probiotic treatment) increased ABN 2-4 during the first postpartum week (O'Leary, 2019). This previous work also showed a trend for probiotic administration increasing LG behaviour (O'Leary, 2019).

The present study found that the probiotic-treated dams engaged in more ABN 2 behaviour specifically than their placebo-treated counterparts, partially consistent with previous findings (O'Leary, 2019). Although my earlier work found that probiotics trended towards increasing LG frequencies (O'Leary, 2019), I did not observe this trend in the current work. Previously, I had hypothesized that this observed increase in ABN 2-4 and trending increase in LG behaviour in the probiotic-treated dams was potentially a result of increased oxytocin (O'Leary, 2019). This hypothesis was based on research showing that administering *Lactobacillus reuteri* ATCC-PTA-6475 to wild-type C57BL/6 mice increased plasma oxytocin levels (Poutahidis et al., 2013). This effect disappeared when these mice were vagotomized but still received probiotic treatment, illustrating that this increase in oxytocin was connected to the gut-brain axis communication (Poutahidis et al., 2013). Since increased ABN 2 levels were still observed in the present study, it is possible that the administration of the probiotic Lacidofil® may be affecting oxytocin levels in the probiotic-treated rats. The observation of increased LG behaviour in WD-fed rats only could potentially be a function of increased satiety in these animals due to the caloric content of their diet, as hypothesized by Kougias and colleagues (2018), allowing them to be in the nest with their pups more. This hypothesis is supported by the reduced number of

feeding and water bouts (F and W, respectively) observed in the WD-fed animals compared to their SD-fed counterparts.

2.1.23 Probiotic Treatment Does Not Impact Inflammation, Metabolic or Stress Markers

Diet-induced obesity's effects on maternal health include higher inflammation levels, compromised placental function and hormone dysregulation (Sullivan et al., 2014). Several interventions have been studied to combat gut dysbiosis, including antibiotics, prebiotics, and probiotics (Cani et al., 2007; Carvalho et al., 2012; Kootte et al., 2012). In the present study, I did not observe any reduction in inflammation levels in the probiotic-treated WD-fed animals, as I had first hypothesized. I did observe that WD-fed animals had increased inflammatory markers, regardless of the function of the analyte (e.g., pro-inflammatory, anti-inflammatory, or regulatory). Lacidofil® has been demonstrated to reduce inflammatory markers in animal models of gastric *H. pylori* infection (Brzozowski et al., 2006; Johnson-Henry et al., 2004). However, there is evidence that *Lactobacillus* treatment is insufficient to reduce inflammation levels (TNF-α, IL-1β, and IL-6) brought on by partial restraint stress in female Wistar rats (Ait-Belgnaoui et al., 2012).

It has been shown that leptin is elevated in obese and diabetic mothers (Hauguel-de Mouzon et al., 2006; Lepercq et al., 1998). In a 16-week high-fat, high-sucrose diet experiment (HFSD; 20% fat, 55% carbohydrates; 20% protein; 5.1 kcal/g) an acute multi-strain probiotic, AVI-5-BAC (*Lactobacillus acidophilus:* 10×10^8 CFU, *Lactobacillus planetarium:* 9.8×10^7 CFU, *Bifidobacterium bifidum:* 2×10^6 CFU, *Bacillus subtilis* fermentation extract: 50 g per kg of product and *Aspergillus oryzae* fermentation extract: 50 g per kg of product) was able to lower leptin levels in male rats (Al-muzafar & Amin,

2017). These results provide evidence for probiotics' ability to regulate the hormonal activity of adipose tissue and demonstrate that probiotic treatment slows weight gain (Al-muzafar & Amin, 2017). While only the HFSD-fed rats showed increased serum levels of TNF-α and IL-6, compared to the control diet rats, the HFSD-fed rats provided an acute probiotic treatment showed lowered levels of these same inflammatory factors, compared to the HSFD-only rats (Al-muzafar & Amin, 2017). This previous work conducted with male rats contrasts with my findings, as I found that the WD-fed female rats had increased plasma leptin and several inflammatory factors with increased expression in plasma. The present study also found no evidence that the probiotic treatment reduced the expression of inflammatory markers or leptin in postpartum females, indicating that there may be sex differences in the effects of probiotic treatment. It is also possible that the dosage of Lacidofil® provided to the rat dams in this study (5×10^8 CFU) was not sufficient to combat the inflammatory effects of the WD. The findings obtained by Al-muzafar and Amin (2017) provided three probiotic strains, each with their own dosage, and two fermentation extracts. Due to these large dosages and varied types of microorganisms, the product provided in this study was perhaps better able to combat the effects of the high-fat, high-sugar diet (Al-muzafar & Amin, 2017)

As previously discussed, estrogen and related compounds may provide some protection regarding metabolic outcomes. Therefore, examining how obesogenic diets impact metabolic markers in female rats is essential. In a study by Nowacka-Chmielewska and colleagues (2021), female rats (unspecified strain) were given a cafeteria diet for six weeks, including candy bars, sausages, crackers, cheese, and potato chips. The obesogenic nature of this diet was furthered by providing the rats with a 10% fructose solution in

addition to their regular drinking water, resulting in a calorically dense diet (4.84 kcal/g, 33.1% fat, 33.2% carbohydrates and 16.6% proteins; Nowacka-Chmielewska et al., 2021). This study found that female rats consuming the obesogenic diet gained significantly more weight than their standard chow-fed counterparts (Nowacka-Chmielewska et al., 2021). Additionally, the obesogenic diet-consuming animals had significantly higher serum leptin levels than the control animals (Nowacka-Chmielewska et al., 2021). A separate study found that WD administration (32% kcal fat diet) resulted in increased plasma leptin and triglycerides in both diet-induced obesity rats and an obesity-resistant rat strain at weaning (Frihauf et al., 2016). These findings are consistent with my study, as I also found that feeding females an obesogenic diet resulted in increased plasma leptin levels.

Although ghrelin is considered an orexigenic hormone, plasma ghrelin levels are typically low in obese people (Tschöp et al., 2001). Indeed, I found similar results in my rat model, where WD-fed dams had lower total plasma ghrelin levels than their SD-fed counterparts.

In addition to disrupting metabolic hormones, as demonstrated by the dysregulation of leptin and ghrelin in the present study, obesogenic diets such as the WD can also disrupt stress hormone levels (Bellisario et al., 2015). Chronic WD consumption in perinatal animal models increases plasma corticosterone, suggesting that WD is a significant physiological stressor during pregnancy (Bellisario et al., 2015). My results show that the WD-fed dams had increased basal plasma corticosterone levels compared to their SD-fed counterparts, consistent with previous findings (Bellisario et al., 2015). Previous research has found that acute Lacidofil® treatment reduced corticosterone levels in rats after they experienced an acute stressor (Ait-Belgnaoui et al., 2012). However, I did not find in my

study, where Lacidofil® was provided for an extended period, that it could mitigate the increase in corticosterone levels observed in the WD-fed animals. It is possible that Lacidofil® can reduce corticosterone levels in an acute stress paradigm but does not reduce basal corticosterone levels found in chronic physiological stressors, such as Western diet administration (Bellisario et al., 2015).

Lastly, due to the relationship between corticosterone and inflammation, I correlated the measures of maternal plasma corticosterone with inflammatory markers. I found that interleukin-1 alpha (IL-1α) levels were negatively correlated with corticosterone levels in the placebo-treated dams. IL-1α enables cytokine activity, copper ion binding, and interleukin-1 receptor binding activity (J. R. Smith et al., 2019). Although IL-1 is involved in HPA axis activation and increases as corticosterone increases, this role is primarily fulfilled by IL-1β specifically (Goshen & Yirmiya, 2009), as demonstrated by comparing a double knockout IL-1α/IL-1β mouse model to single knockout IL-1α, IL-1β or IL-1 receptor antagonist mice (Horai et al., 1998). It is unclear why IL-1α levels would negatively correlate with corticosterone levels in the present study. This relationship between IL-1β and the activation of the HPA axis is observed in the significant positive correlation between IL-1β and corticosterone levels in all animals included in this study.

Growth Related Oncogene/Keratinocyte Chemoattractant (GRO/KC; human homolog CXCL1) facilitates chemokine activity and participates in neutrophil chemotaxis (J. R. Smith et al., 2019). GRO/KC is released in response to acute injury or stress, such as LPS injections (N. Li et al., 2009; Shibata et al., 1996). GRO/KC levels were positively correlated with corticosterone levels in the probiotic-treated dams. The amount released is typically proportional to the stressor's intensity (Campbell et al., 2003). Due to the

association between acute stress and GRO/KC release, the positive correlation observed is understandable. However, it is unclear why this association would only be present in the probiotic-treated dams. Recent research on the effects of probiotics on human colonic cells demonstrated that probiotic administration induced a mild inflammatory response, including the upregulation of GRO/KC mRNA expression (Tarapatzi et al., 2022). Due to the low-grade inflammatory state induced by the probiotic administration, the authors postulated that the probiotics may play an immune system alerting role, as they do not appear to cause a prolonged inflammation response (Tarapatzi et al., 2022). Similarly, studies of human peripheral mononuclear blood cells treated with probiotics have shown that probiotic administration does up-regulate the expression of several cytokines, including the human homolog of GRO/KC, CXCL1, as well as TNF-α, IL-1β, and IL-6 (all pro-inflammatory cytokines; (Vale et al., 2023).

Macrophage Inflammatory Protein 3 alpha (MIP-3α; human homolog CCL20) responds to molecules of bacterial origin and enables chemotaxis and chemokine activity (J. R. Smith et al., 2019). I found that MIP-3α levels were negatively correlated with corticosterone for the standard diet-fed dams only. This result is consistent with previous work demonstrating that corticosterone administration reduced MIP-3α (CCL20) mRNA expression in a rat cell line (Burke et al., 2017).

2.1.24 Limitations

This study had several limitations. First, the researchers could not be blinded to the animals' treatment and diet conditions due to reduced staffing because of the COVID-19 pandemic. Second, fecal samples were not taken before diet/probiotic administration or

collected throughout the experiment. Instead, the animals' caeca were taken at sacrifice. I did not do an oral glucose tolerance test or take blood samples throughout the experiment; I only took them at sacrifice, as due to COVID-19 restrictions during this study, conducting tail blood draws from rat dams was not feasible. Food was provided *ad libitum,* and WD animals did show markers of obesity, such as increased weight gain after six weeks of diet administration and dysregulation of metabolic hormones. Although, for translational research purposes, this does not necessarily mimic human food consumption patterns. Due to the small number of animals per experimental treatment group, I could not conduct correlations at this level. As a result, the correlations between maternal plasma corticosterone and inflammation levels do not provide a complete picture of how probiotic treatment, combined with Western diet, may affect one another.

2.1.25 Implications and Future Directions

This work adds to the literature on Lacidofil® concerning its effects on inflammatory and metabolic markers, the stress hormone corticosterone in a rodent maternal health model, and its impact on maternal care. Future work should investigate how other probiotic strains, such as inflammation, metabolic markers, and stress, may impact maternal care and health outcomes. Additionally, given the contradictory results regarding how different diet manipulations impact maternal care, future studies should investigate other diets' effects on maternal behaviour. Future studies could take blood samples across the perinatal period, as this would be beneficial for characterizing any differences in hormones (e.g., estrogen, progesterone, corticosterone) that may occur due to diet or probiotic administration.

For this study, future avenues of investigation may include metabolomic analyses of stomach contents to assess how Lacidofil® and diet may interact to make distinct metabolite profiles in the gut. Further, to begin to probe the mechanisms by which both Lacidofil® and the Western diet impact maternal care behaviour expression, performing RNA sequencing on brain regions involved in maternal care expression would provide an excellent foundation for the underpinnings of these changes.

Overall, there needs to be more information regarding how internal and external factors, such as diet and probiotic administration, impact maternal outcomes.

CHAPTER 3 THE EFFECTS OF DIET AND PROBIOTIC ADMINISTRATION ON MATERNAL GUT MICROBIOTA COMPOSITION

INTRODUCTION

There is increasing interest in how the maternal gut microbiota (i.e., the microorganisms living within the intestinal tract) may affect maternal health (Dunlop et al., 2015). Of note, changes in maternal hormones, metabolism and inflammation can affect the composition of the gut microbiota during pregnancy (Koren et al., 2012; Mallott et al., 2020; Nuriel-Ohayon et al., 2019). For instance, research suggests that increased estrogen and progesterone as a consequence of pregnancy can alter the microorganisms that are present in the gut microbiota (García-Gómez et al., 2013). Furthermore, pregnancy-associated hormonal and microbial changes may increase nutrient intake to help maintain the pregnancy. Namely, these microbes can produce metabolites (e.g., short-chain fatty acids [SCFAs] like 5-amino valeric acid betaine and trimethylamine N-oxide) that help a pregnancy host convert nutrients into usable energy (Barrientos et al., 2024; Pessa-Morikawa et al., 2022).

3.1.1 The Gut Microbiota During Pregnancy

Significant changes to the gut microbiota composition occur between the first and third trimesters of a human pregnancy (Koren et al., 2012). Specifically, a decrease in species richness (alpha diversity: the richness of bacteria in the sample) and an increase in species diversity (beta diversity: the distance between samples as a measure of how similar or different they are) have been reported between the first and third trimesters (Koren et

al., 2012). An increase in beta diversity was also observed in a study where the gut microbiome was sampled at 32 weeks and again just before parturition (Y. Chen et al., 2019).

As the mother's inflammation profile changes as gestation progresses, the microbiome is not only affected by said inflammation but also assists in its regulation (Koren et al., 2012). Approximately 70% of pregnant women have a greater abundance of inflammation-associated bacteria (Santacruz et al., 2010). For example, women have an increased abundance of Proteobacteria during the third trimester, associated with a pro-inflammatory state (Mukhopadhya et al., 2012; Rizzatti et al., 2017). This crosstalk between the maternal immune system and the microbiome is an important aspect of a healthy pregnancy (Barrientos et al., 2024; Koren et al., 2012), as the pro-inflammatory state during the third trimester is an important preparatory step for parturition (Mor et al., 2017).

3.1.2 Probiotic Administration and the Gut Microbiota During Pregnancy

Nutritional supplements, such as probiotics, are of particular interest in microbiome research (e.g., probiotic strains can produce these aforementioned SCFAs; Ljungh & Wadstrom, 2006). A recent study on probiotic administration in a mouse pregnancy model shows promising results: probiotics may help regulate the maternal immune system (Tao et al., 2020). Researchers found that probiotic administration of a beneficial *Enterococcus faecalis* strain, Symbioflor1, to pregnant mice affected by the opportunistic pathogenic strain *Enterococcus faecalis* OG1RF, reduced miscarriage rates and upregulated the anti-inflammatory cytokine interleukin 10 (IL-10; Tao et al., 2020).

Largely, human research suggests that, similar to studies using non-pregnant participants, probiotic administration does not cause large shifts in microbiome composition during pregnancy (Bisanz et al., 2015; Y. Chen et al., 2019). For example, Tanzanian women (second trimester of pregnancy) who were given a daily serving of probiotic yogurt (*Lactobacillus rhamnosus* GR-1 at approximately 1×10^{10} CFU with added Moringa leaf) did not have significant changes in their gut microbiota compared to an untreated control group of pregnant women (Bisanz et al., 2015). Likewise, in a placebo-controlled study of healthy pregnant women taking a multi-strain probiotic (*Bifidobacterium longum* [5×10^6], *Lactobacillus debrueckii bulgaricus* [5×10^5], and *Streptococcus thermophilus* [5×10^5]), researchers reported no compositional fecal gut microbiota differences (e.g., alpha or beta diversity metrics), nor any differences in the relative abundance of the specific microorganisms present in the fecal samples (Y. Chen et al., 2019).

Despite studies reporting that specific probiotics do not impact the composition of the gut microbiota, there is still evidence that probiotics can provide some health-related benefits to the host during pregnancy. A systematic review on the impacts of probiotic yogurt on maternal health and pregnancy reported reduced occurrence of preterm birth and maintenance of serum insulin and calcium levels in those given the yogurt compared to study controls (A. He et al., 2020). In another study, pregnant women were provided with a combination prebiotic (inulin fibre) and probiotic supplement (i.e., 10 different probiotic strains that were predominantly *Lactobacillus* strains; 1×10^{10} CFU total) for 12 days (A. T. Liu et al., 2021). Although no changes in fecal microbiota were reported in this study (e.g., alpha diversity metrics, relative abundance of specific bacteria) following probiotic

treatment (compared to pre-probiotic treatment), probiotic administration did reduce pre-treatment self-reported gastrointestinal symptoms (e.g., nausea, vomiting and constipation) compared to the participants' pre-probiotic treatment symptoms (A. T. Liu et al., 2021).

3.1.3 Western Diet and the Gut Microbiota During Pregnancy

An organism's diet greatly affects gut microbiome composition (Connor et al., 2023; David et al., 2014). In turn, the gut microbiota affects how organisms store and utilize energy from food (Tremaroli & Bäckhed, 2012). Broadly, alterations in the gut microbiota during pregnancy linked to diet are observed in mice (Connor et al., 2018), primates (Sugino et al., 2022), and humans (Gomez-Arango et al., 2016). Of note, Western diet (WD) administration in animal models during pregnancy results in gut microbiota and placental changes, along with changes in oxidative stress and inflammation (Gohir et al., 2019; Y.-W. Wang et al., 2021). For example, pregnant baboons fed a WD had a significantly lower species richness (alpha diversity metric) than the control diet-fed baboons (Sugino et al., 2022). Another study with pregnant C57BL/6 mice fed a high-fat diet (HFD) showed lower species richness in their gut microbiota than control diet-fed mice (Connor et al., 2018).

In overweight and obese women, significant positive correlations have been reported between adipokine levels (e.g., leptin) and the abundance of the families Ruminococcaceae and Lachnospiraceae (Firmicutes phylum) (Gomez-Arango et al., 2016). Greater abundance of the families Bacteroidaceae (Bacteroidetes phylum) may also be linked with maternal metabolism (G. Jiang et al., 2023), as increased Bacteroidaceae positively correlates with the metabolic hormone ghrelin (Gomez-Arango et al., 2016). In

fecal samples from pregnant women, the *Collinsella* genus was reported to be positively correlated with insulin and triglyceride levels (Gomez-Arango et al., 2016). Additional evidence from other studies suggests that WD consumption during pregnancy may produce an altered gut microbiota composition (e.g., lower species richness; Nagpal et al., 2018; Röytiö et al., 2017).

3.1.4 Objectives and Hypotheses

Microbial differences exist between human and rat gut microbiomes (Nagpal et al., 2018). Nonetheless, rats offer some benefits as a model system because their digestive tract is similar to that of humans, with both having comparable stomach and intestinal structures (Vdoviaková et al., 2016). Furthermore, the gut microbiota of both rats and humans contains an abundance of bacteria from the phyla Bacteroidetes and Firmicutes (Nagpal et al., 2018).

This chapter contains two studies of rat gut microbiota. Study 1 was part of a larger project examining probiotics as a supplement for healthy-weight dams during pregnancy and lactation (42 days of probiotic administration). Study 2 was also part of a larger project examining the effects of a WD and probiotic administration from six weeks before conception through pregnancy and ending in the second postpartum week (11 weeks of diet and probiotic administration). The rationale for both studies is that probiotic administration throughout gestation could minimize dramatic microbiota shifts during this time and perhaps bolster the microbial community.

For Study 1, I was interested in the effects of probiotic administration with the probiotic Lacidofil® (*Lacticaseibacillus rhamnosus* R0011 and *Lactobacillus helveticus*

R0052) on the dams' gut microbiota composition. It was hypothesized that Lacidofil® treatment would alter the dams' microbiota such that it would differ from the placebo-treated dams; however, there were no specific hypotheses about these alterations. For Study 2, I was interested in how probiotic treatment with Lacidofil® would interact with WD administration across the perinatal period to impact the gut microbiota composition. It was hypothesized that WD administration would have a larger impact on the gut microbiota (i.e., affecting a larger number of measures) than Lacidofil® administration. However, based on previous studies, I hypothesized that Lacidofil® would likely impact a few gut composition measures.

METHODS

3.1.5 Study 1: Animals, Breeding and Probiotic Administration

This experiment was conducted using caecum samples obtained from 15 female Long-Evans rats (Charles River, St. Constant, QC, Canada) who were exposed to either placebo (n = 7) or Lacidofil® (n = 8) treatment throughout pregnancy and nursing (a total of 47 treatment days). The experimental procedures were approved by the Dalhousie University Committee on Laboratory Animals (UCLA) under animal ethics protocol #18-022 and performed in accordance with the Canadian Council on Animal Care guidelines (CCAC).

Rats were housed in same-sex pairs in polypropylene cages with wire lids. The cages had animal bedding (Fresh Bed, Shaw Resources, Shubenacadie, NS, Canada) and a black polyvinyl chloride tube. Food (Laboratory Rodent Diet 5001, LabDiet®, St. Louis, MO, United States of America) and tap water were provided *ad libitum.* Male and female

rats were paired for breeding for 7 days. After the breeding period, dams were provided a probiotic or placebo solution from the first day of the gestation period until their offspring were weaned at 21 days old (see Figure 3.1A for experimental timeline).

Dams were trained to receive probiotic or placebo solution orally via syringe as per Tillman & Wegner, 2018, with minor modifications as detailed by Myles and colleagues (2020). The probiotic solution was prepared at a dosage of 500 million (10^8) colony-forming units (CFU) per 0.5 mL of reverse-osmosis water daily. Each rat was given 0.5 mL of the appropriate solution (0.0192 grams of powder).

3.1.6 Study 1: Caecum Sample Collection

Dams were sacrificed five days after their offspring were weaned and within 0-8 hours of receiving treatment. They were first deeply anesthetized with Euthanyl® (sodium pentobarbital, 60 mg/kg, intraperitoneal). Caeca were quickly dissected and flash-frozen whole on dry ice. The caeca were stored at -80ºC until the contents could be aliquoted into microtubes for later use.

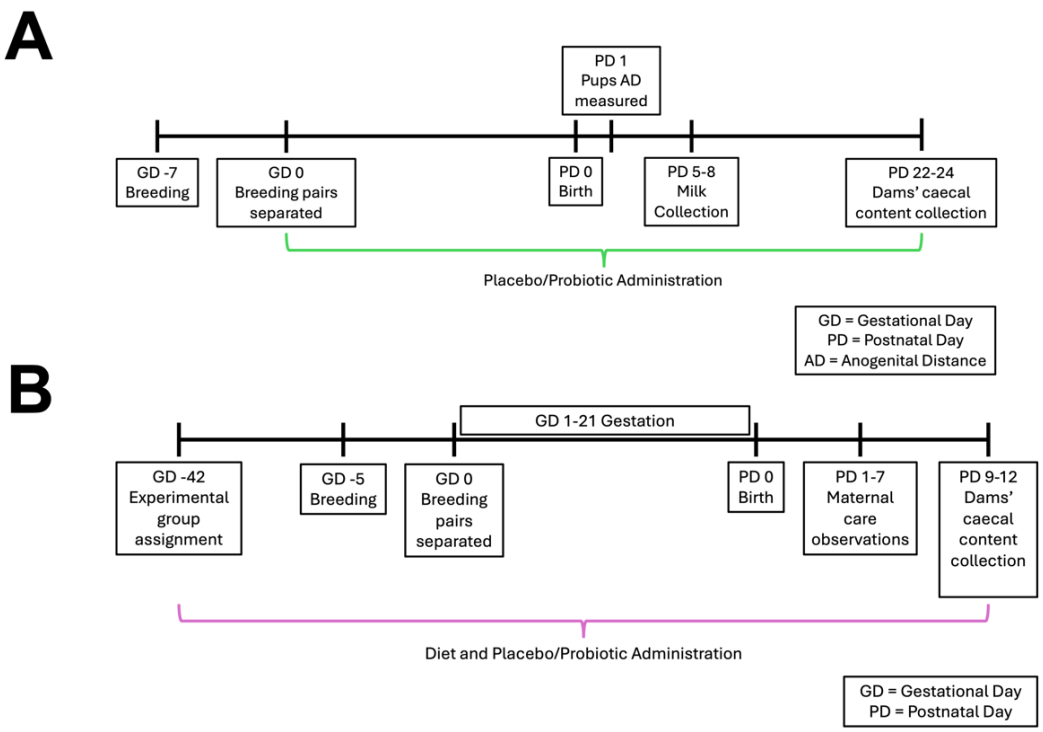

Figure 3.1 A) An experimental timeline illustrating Study 1. B) An experimental timeline illustrating Study 2.

3.1.7 Study 2: Animals, Breeding and Probiotic Administration

The experiment for Study 2 was conducted under animal ethics protocol #20-136, as approved by the Dalhousie University Committee on Laboratory Animals (UCLA) and the Canadian Council on Animal Care guidelines (CCAC).

The animals utilized for this study are the same female rats described in Chapter 2 of this thesis. Forty female Long-Evans rats were purchased from Charles River Laboratories and split evenly between a placebo and a probiotic treatment colony room. This experiment's colony room and cage conditions are the same as those for Study 1. After a two-week quarantine, the females were randomly assigned a diet condition. The diet (Standard or Western) and treatment (placebo or probiotic) administration started the day

after the quarantine period ended (see Figure 3.1B for an experimental timeline). There were four experimental groups: Placebo-Standard Diet (n = 8), Placebo-Western Diet (n = 7), Probiotic-Standard Diet (n = 8) and Probiotic-Western Diet (n = 8).

The Western diet (cat. #D12079B, Research Diets, New Brunswick, NJ, USA) and the standard diet (Laboratory Rodent Diet 5001, LabDiet®, St. Louis, MO, United States of America) were both provided *ad libitum* (see Appendix A for the diet information sheets).

The placebo and probiotic treatments were provided using an oral syringe, following Tillmann and Wegener's (2018) protocol. To prevent cross-contamination between the placebo and probiotic-treated animals, I employed the protocols outlined by Myles and colleagues (2020). The probiotic used in this study was Lacidofil® (*Lacticaseibacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052; see Table 3.3; Foster et al., 2011).

Table 3.1 Placebo and probiotic (Lacidofil®) vehicle formulation

Ingredient	% of Formulation	Amount in Grams
Maltodextrin	85	425
Magnesium Stearate	3	15
Milk Powder	11.6	58
Ascorbic Acid	0.4	2

3.1.8 DNA Protocols

All protocols for processing and handling the caecal content DNA were the same between Study 1 and Study 2.

3.2.4.1 DNA Extraction

Caecum DNA Extraction. DNA was isolated from dams' caecum samples with the QIAamp® DNA Stool Mini Kit (Cat. #51504, QIAGEN, Germany). The samples underwent an initial pre-processing step and were then processed according to the manufacturer's instructions. First, an average of 257.14 mg of each caecum sample was weighed and placed into individual bead-beating tubes containing four 3mm glass beads (Fisher Scientific, Cat. 11-312A). 500 µL of phosphate-buffered saline (PBS; ThermoFisher, Cat. #10010023) was added to each tube, and the samples were vortexed thoroughly. An additional 500 µL of PBS was added, and samples were vortexed again. Then, the samples were centrifuged for 1 minute at 11,500 × *g*. Afterwards, the supernatant was discarded, and the pellet was washed using 1 mL of PBS. An additional centrifugation was performed for 1 minute at 11,500 × *g,* and the supernatant was discarded. 1 mL of InhibitEX was then added to each sample, homogenized via vortexing, and incubated at 70^{o}C in a dry bath for 5 minutes.

Post-incubation, approximately 300 mg of zirconia beads were added to each sample, followed by homogenization via a bead homogenization protocol (MP FastPrep-24^{TM} 5G, Cat. #116005500). The protocol consisted of three rounds of 60-second homogenization at 4 m/s, with a 60-second rest period in between each bead-beating step. After homogenization, the samples were centrifuged for 3 minutes at 11,500 × *g*. Then, 600 µL of the supernatant was transferred into a new, labelled microtube and centrifuged for 3 minutes at 11,500 × *g*.

The next step was to add 25 µL of Proteinase K to a new microtube and 400 µL of the supernatant. Once the sample was transferred, 400 µL of Buffer AL was added,

followed by vortexing for 15 seconds. The samples were then incubated in a dry bath at 70°C for 10 minutes. Post-incubation, 400 µL of 100% ethanol was added to the lysate and vortexed. 600 µL of lysate was then transferred onto a spin column and centrifuged at 14,100 × *g* for 1 minute. The flow-through was discarded after centrifugation, and 600 µL of lysate was added to the column again, transferring all the lysate, then centrifuging again at 14,800 × *g*. Next, 500 µL of Buffer AW1 was pipetted onto the spin column, and the samples were centrifuged at 14,100 × *g* for 1 minute. Then, 500 µL of Buffer AW2 was pipetted onto the column and centrifuged at 14,100 × *g* for 1 minute. The column was transferred onto a new 2 mL microtube, and 50 µL of Buffer ATE was pipetted directly onto the column membrane. All samples were incubated for 5 minutes at RT and centrifuged at 14,100 x *g* for 1 minute to elute the DNA. Once the extraction was complete, the DNA concentration (ng/µL) and purity (260/280 ratio) of each sample were obtained using spectrophotometry. All the DNA samples were stored at -20°C until further analysis.

DNA Normalization. Using the DNA concentrations obtained from spectrophotometric readings, I calculated the volume of DNA (µL) and the volume of nuclease-free water needed to obtain a final concentration of 20 ng/uL. These values were computed using Microsoft Excel, transferred to a .csv file and uploaded to the *epMotion*® software to program a liquid handling robot (ep*Motion*® 5075t, Eppendorf, Hamburg, Germany) to perform the dilutions. Once the dilutions were complete, they were stored at -20°C until further analysis.

Sample Spiking. The following protocol is from the standard operating procedure (SOP) No. CLIN-0017 from Lallemand Health Solutions, Inc. This protocol aims to add a known number (10^{10}) of bacterial cells to a control caecal content sample. The DNA is

extracted using the same process as the unknown samples. Then, it is serially diluted to make a standard curve to quantify the probiotic strains' presence (LOG CFU per gram) in the samples.

First, I began by weighing 3.84 grams of control caecal samples obtained from eight control male rats into a sterile 5 mL microtube to create a spiking matrix. The matrix was homogenized manually and then separated as equally as possible (the average amount of caecal contents per tube was 320 mg) into 12 sterile 2 mL bead beating tubes containing four sterile 3 mm glass beads.

Then, for each probiotic strain (R0011, R0052 and R0175), one gram of the probiotic powder was placed into a Falcon tube containing 9 mL of PBS to create a 1:10 dilution for each strain. This solution was vortexed thoroughly to mix. I calculated how many µL needed to spike in a 10^{10} CFU concentration for each strain and then pipetted that amount into the designated bead-beating tubes of caecal contents (each strain had a total of three spiked tubes and one unspiked; see Appendix E for sample spiking calculations). The spiked samples were mixed thoroughly once the calculated amount of spiking solution was added to the correct tubes. Then, the DNA extraction proceeded as outlined above for the caecal content samples.

Spiked Sample Testing via Real-Time Quantitative PCR (qPCR). After the DNA was extracted and the concentrations (ng/µL) and purities (260/280 ratio) were checked using spectrophotometry, the next step was to validate the spiked samples to select which sample would be used to make the standard curve for each strain. The 10^{10} stock DNA for each spiked sample was serially diluted in molecular-grade water to obtain concentrations from 10^{9} to 10^{3}. The serial dilution was done by adding 2 µL of the stock

to 18 µL of molecular grade water in a new microtube, vortexing thoroughly to make the 10^9 dilution, and then taking 2 µL of that dilution and adding it to a new microtube with 18 µL of water and so on. It is crucial to thoroughly mix each tube in a serial dilution, so to ensure this, I pipetted each dilution up and down 20 times, vortexed briefly three times, spun the tube down, vortexed again three times briefly and once again spun down to collect the tube's entire contents.

Next, to set up a qPCR assay, three separate Master Mixes were made using SYBR green chemistry (1x SYBR Select Master Mix, Cat. #4472980; Life Technologies, Carlsbad, USA), the Master Mix Calculation (see Appendix F for qPCR Master Mix calculations) and 300 nM strain-specific primers (Myles, O'Leary, Romkey, et al., 2020) to assay all spiked sample standard curves and the undiluted unspiked samples. A 96-well plate was prepared by adding 2.5 µL of template DNA to 22.5 µL of Master Mix. All samples for each probiotic strain were run in triplicate, including the unspiked samples and a no template control (containing only Master Mix and molecular-grade water). The plate was sealed with MicroSeal® optical adhesive film (Bio-Rad Laboratories, Hercules, USA), briefly vortexed and centrifuged at 2,000 RPM for 1 minute before putting the plate into the real-time PCR machine. The qPCR assay was run using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with the following cycling conditions: 50°C for 2 minutes, 2-minute hold at 95°C followed by 40 cycles of denaturation for 15 seconds at 95°C, annealing for 30 seconds at 60°C and an extension or 30 seconds at 72°C. Afterwards, the amplification curves for each spiked sample were checked using the CFX Maestro™ software (Version 4.1, Bio-Rad). I chose the spiked sample with all three amplification curves overlaying one another, ensuring an accurate standard curve. The sample with the

tightest replicates was selected to create the standard curve for the strain detection experiment. I also checked the unspiked samples to ensure that there was either no amplification or that any amplification was greater than 34 quantification cycles (Cq).

Strain Detection via Real-Time Quantitative PCR (qPCR). This experiment was performed according to the protocol outlined in Myles et al., 2020. The DNA of all dams' caeca content was first diluted to a 1:5 ratio in molecular-grade water using a liquid handling robot (ep*Motion*® 5075t, Eppendorf, Hamburg, Germany).

Next, the standard curve was created using the selected spiked sample from the previous spiked sample testing step. The standard curve was created using the liquid handling robot to serially dilute this 10^{10} sample 10-fold down to 10^{3}. All samples, standards, and controls (unspiked and no-template controls) were then loaded onto a 384-well plate via the robot, using 9 µL of Master Mix and 1 µL of DNA or control. The samples were run in triplicate, and the standard curve was run in duplicate. The qPCR assay was run on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad) with the following cycling conditions: 50°C for 2 minutes, 2-minute hold at 95°C followed by 40 cycles of denaturation for 15 seconds at 95°C, annealing for 30 seconds at 60°C and an extension or 30 seconds at 72°C. After 40 cycles, a dissociation curve from 60 to 95°C was done to check the amplification specificity of the primers. All data analysis was performed using the CFX Maestro™ software (Version 4.1, Bio-Rad).

3.2.4.2 Microbiota Profiling Using 16S rRNA Sequencing

Using the caecal content DNA that was previously normalized to 20 ng/µL for the strain detection experiment, 16S rRNA sequencing was performed. The protocols for the microbiota profiling and subsequent data analysis are the same for Study 1 and Study 2.

Amplicon PCR. The first PCR reaction utilized the following reagents and reaction volumes to prepare a Master Mix (see Appendix G for amplicon PCR Master Mix calculations). The primers were first diluted to 1 µM with a 1:100 dilution in PCR-grade water (see Appendix G for 16S rRNA primer). Into a 96-well plate, 2.5 µL of template DNA was added to each well, followed by 22.5 µL of master mix, resulting in 25 µL per well. Once it was loaded, the plate was sealed and run at the following cycling conditions: 95°C for 3 minutes, 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes then hold at 4°C.

Gel Electrophoresis. First, the mixture of 6x NEB loading dye (300 µL) and PCR water (700 µL) was prepared in a 1.5 mL microtube. A 96-well plate was then prepped by loading 5 uL of the dye mixture into each well using a multichannel pipette. Then, 5 µL of each amplicon from the amplicon PCR was loaded into the 96-well plate, as per the sample template. Using an E-gel pre-cast 2% agarose gel with SYBR® Safe DNA gel stain, the 10 µL sample and dye mixture were first mixed using the multichannel pipette, and then all 10 µL was transferred into the gel. At the end of the gel, a 2-log ladder was loaded. The gel was run for a minimum of ten minutes and then visualized using the Bio-Rad® Gel Imaging Doc, using the SYBR® Safe option.

PCR Clean-Up #1. The PCR amplicon plate was centrifuged at 1,000 x *g* at room temperature for 1 minute to ensure all the contents were at the bottom of the wells. The samples (25 µL) were transferred to a MIDI (deep well) 96-well plate via a multichannel pipette for the clean-up step. Before adding the AMPure XP beads, the beads were vortexed for 30 seconds before 20 µL of beads per sample were added to the plate using a multichannel pipette. After adding the beads, the plate was sealed and shaken at 1,800 RPM

for 2 minutes. Following the shaking step, the plate was then incubated at room temperature for 5 minutes. After this RT incubation, the plate was put on a magnetic stand for 2 minutes. The plate was then kept on the magnetic stand and a multichannel pipette was used to remove the supernatant, changing tips between samples.

After removing the supernatant from all wells, the magnetic beads were then washed using freshly prepared 80% ethanol (200 µL per sample). The plate was incubated for 30 seconds (remaining on the stand) and then the supernatant removed. An identical ethanol wash and incubation was then performed. After this second wash, a P20 multichannel was used to remove access ethanol. The beads were then air-dried for 10 minutes.

The PCR plate was removed from the magnetic stand, and 52.5 µL of 10 mM Tris (pH 8.5) was added to each well. To mix, the plate was sealed and shaken at 1,800 RPM for 2 minutes to resuspend the beads. Following the shaking step, the plate was incubated at room temperature for 2 minutes and placed back on the magnetic stand for 2 minutes. With a multichannel pipette, 50 µL of the supernatant from each well was transferred to a new 96-well plate.

Index PCR. With a multichannel pipette, 5 µL from each well was transferred into a new 96-well plate. The remaining sample was stored at -20ºC for potential future use. A Master Mix containing two indexing primers was added to each well (see Appendix G for Master Mix reagents and calculation table). Reagents were pipetted up and down ten times to mix and the plate was sealed and centrifuged at 1,000 x *g* at 20ºC for 1 minute. The plate was then put into a thermal cycler with the following cycling conditions: 95°C for 3

minutes, 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes, then hold at 4°C.

PCR Clean-Up #2. In this PCR clean-up, AMPure XP beads were used to clean the final library before quantification. First, the Index PCR plate was centrifuged at 280 x *g* RT for 1 minute. The AMPure XP beads were vortexed for 30 seconds before adding the beads to a reservoir. For each well, 56 µL of the bead solution was added to the Index PCR plate, and wells were mixed by carefully pipetting up and down 10 times. The plate was then incubated at room temperature for 5 minutes. After the incubation, the plate was placed on a magnetic stand and left for 2 minutes to clear the supernatant. Once the supernatant appeared clear, the supernatant was discarded via a multichannel pipette. The Index PCR plate was left on the magnetic stand to wash the beads with 80% ethanol. Following the first wash, a second wash with 80% ethanol was performed, ensuring that all excess ethanol was removed using a pipette. After the second wash, the beads were air-dried for 10 minutes. Then, the plate was removed from the stand, and 27.5 µL of 10 mM Tris pH 8.5 was added to each well and mixed by pipetting up and down to resuspend the beads. The plate was then incubated at room temperature for 2 minutes and then placed on the magnetic stand again for 2 minutes to clear the supernatant. Lastly, 25 µL of the supernatant from each well was transferred to a new 96-well PCR plate.

Library Quantification, Pooling and Normalization. The DNA library quantification was done using a fluorescence assay (Quant-iT PicoGreen dsDNA Assay Kit, ThermoFisher Scientific, Cat. # P7589) according to the manufacturer's instructions. Once measured, 200 ng of DNA from each sample was taken from the 96-well microplate and transferred into one 1.7 mL microtube via a liquid handling robot. Then, a fluorometer

(Qubit™ 1X dsDNA Broad Range Assay Kit, Cat. #Q33265) was used to determine the concentration of the pooled sample. Once the concentration was calculated, the pooled sample was diluted to 1 ng/μL and measured via a fluorometer to ensure accurate loading calculations for the Tape Station and MiSeq applications.

Tape Station. To ensure that the samples' amplification was of good quality and specific, the Agilent D1000 ScreenTape Assay (Agilent, Cat. #5067-5583) was performed according to the manufacturer's protocol. The estimated amplicon sizes from the assay were then used for MiSeq loading concentration calculations.

Library Denaturation and PhiX Control. The pooled library and the PhiX internal sequencing control were denatured using NaOH. First, 5 μL of the pooled library was added to 5 μL of 0.2 N NaOH (diluted from 1 N NaOH) and allowed to denature for 5 minutes at RT. Then, 990 μL of HT1 was added to the 10 μL of denatured DNA. Similarly, 2 μL of 10 nM PhiX stock was added to 2 μL of 0.2 N NaOH and denatured for 5 minutes at RT. Then, the 4 μL of denatured PhiX was added to 996 μL of HT1. Next, a 5% PhiX solution was mixed using the denatured pooled library and denatured PhiX for use in the MiSeq run.

MiSeq Run. Amplicon sequencing of the V3-V4 region for the 16S rRNA gene was performed using an Illumina Miseq, according to the manufacturer's instructions.

3.1.9 Bioinformatic Analyses

The Microbiome Helper repository was used as a guide to process the sequencing data (Comeau et al., 2017). First, the raw reads were imported to QIIME2 v2022.2 (Bolyen et al., 2019) and the primers were removed using the Cutadapt QIIME2 plug-in (M. Martin,

2011). The primer sequences used for the 16S rRNA gene V3-V4 region can be found in Appendix G. The trimmed reads were summarized and denoised using deblur (Amir et al., 2017) and trimmed to 450 base pairs. Amplicon sequence variants (ASVs) were identified using the Scikit-learn (Pedregosa et al., 2011) naïve Bayes classifier, trained on the full-length 16S rRNA gene reference database SILVA (Version 38; Quast et al., 2012). Rare (i.e., present in only one sample) and low-abundance taxa (i.e., ten or fewer reads per sample) were subsequently removed, as well as taxa that were not classified at the phylum or domain level or were classified as mitochondria or chloroplasts. The sampling depth was assessed through rarefaction curve visualization. Further bioinformatic analyses were conducted using R (Version 4.3.1.; (R Core Team, 2021) and RStudio (Version 2023.06.1.52; *RStudio: Integrated Development Environment for R.*, 2023) with the following packages: phyloseq (McMurdie & Holmes, 2013), vegan (Oksanen et al., 2013), MaAslin2 (Mallick et al., 2021) and ALDEx2 (Fernandes et al., 2013).

RESULTS

3.1.10 qPCR Strain Detection for Study 1

This work was previously summarized in (Myles, O'Leary, Romkey, et al., 2020). Briefly, no probiotic strains were detected in the placebo animals, and both probiotic strains, *L. rhamnosus* R0011 and *L. helveticus* R0052, were detected in the probiotic animals.

3.1.11 Microbiota Analyses for Study 1

3.3.2.1 Species Richness (Alpha Diversity)

I looked at the following metrics for alpha diversity to ensure that the sample distribution looked approximately the same for each: Observed Features, Chao, Shannon, and Simpson. One sample in the probiotic-treated group was found to have low alpha diversity across all metrics, so that sample was removed before statistical analyses. I used the Observed Features, Shannon, and Simpson metrics for my statistical analyses. I found no differences in these metrics between placebo- and probiotic-treated animals.

3.3.2.2 Species Diversity (Beta Diversity)

When analyzing beta diversity, I looked at the following metrics using permutational multivariate ANOVAs (PERMANOVAs): Weighted Unique Fraction Metric (UniFrac), Unweighted UniFrac, Aitchison's Distance and Phylogenetic Isometric Log-Ratio Transform (PhILR). I found no differences in species diversity (Weighted UniFrac) between the placebo- and probiotic-treated dams ($F = 1.30$, $p = .206$, $r^2 = .09$). The Unweighted UniFrac distances significantly differ between the placebo- and probiotic-treated dams ($F = 1.39$, $p = .035$, $r^2 = .10$). I found a significant difference in species diversity based on Aitchison's Distance ($F = 7.61$, $p < .001$, $r^2 = .37$; see Figure 3.2).

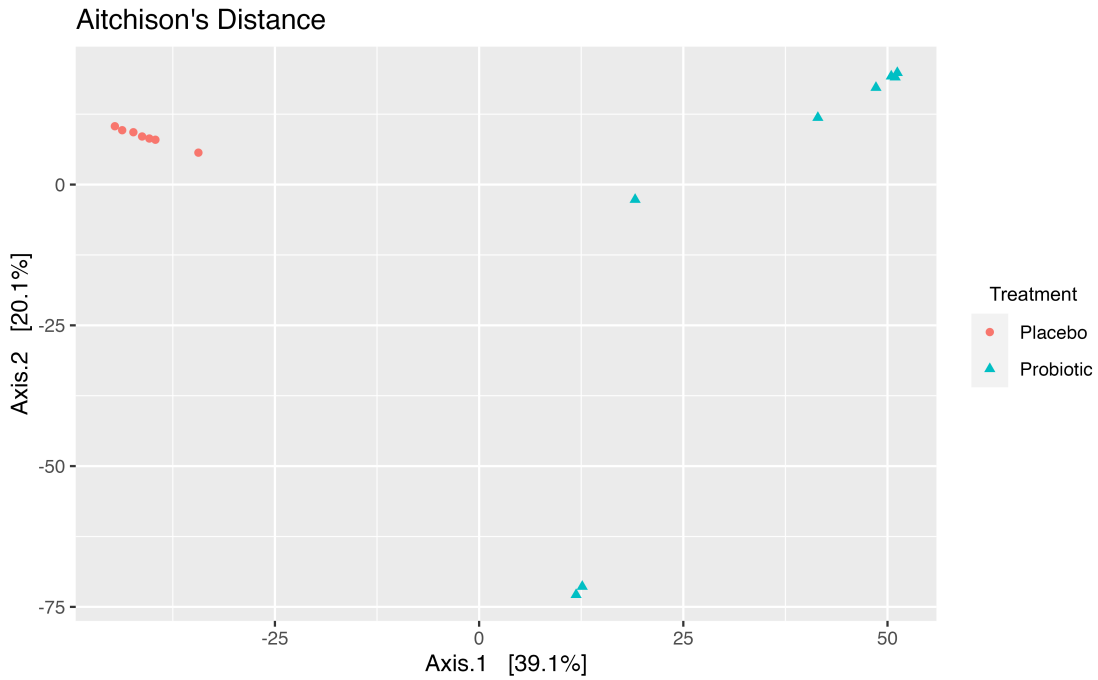

Figure 3.2 This figure illustrates the species diversity metric, Aitchison's Distance, showing the significant main effect of treatment. Aitchison's Distance is calculated using Euclidean distance (i.e., the length of a line between two points), which is robust to sub-compositions of the data (Gloor & Reid, 2016).

I also found a significant difference between the species diversity in the microbiota of placebo- versus probiotic-treated animals when I analyzed PhILR values ($F = 11.09$, $p < .001$, $r^2 = .46$; see Figure 3.3).

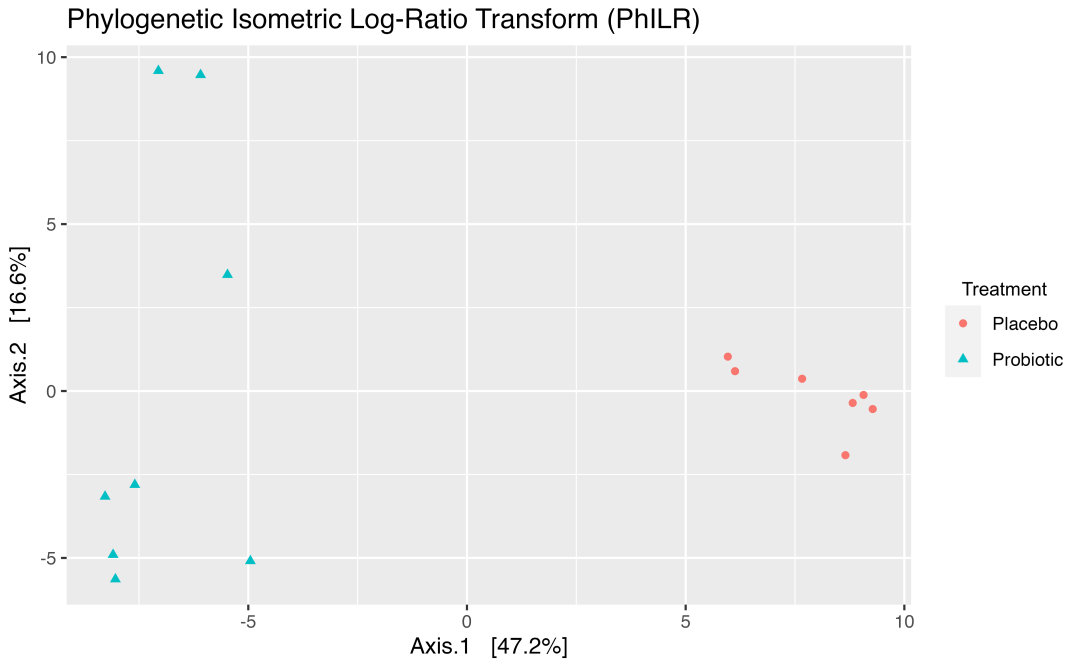

Figure 3.3 This figure illustrates the species diversity metric, PhILR, showing the significant main effect of treatment. The PhILR metric uses Euclidean distance (i.e., the line length between two points) and weights the data to account for zero or near-zero counts and phylogenetic branch lengths (Silverman et al., 2017).

3.3.2.3 Relative Abundance

I examined the relative abundance of features at the phylum (see Figure 3.4) and genus levels (see Figure 3.5).

At the phylum level, I observed little variation across the placebo and probiotic-treated groups. I also observed high variability between individual animals at the genus level, regardless of treatment.

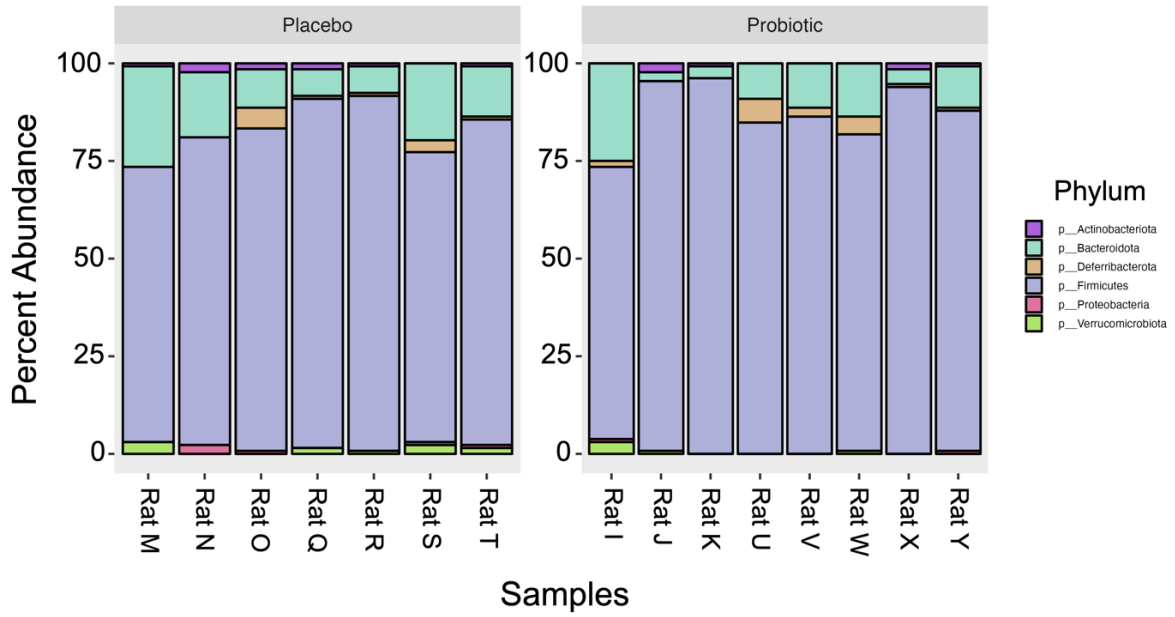

Figure 3.4 This figure shows the top six most abundant phyla (L = 2) by treatment.

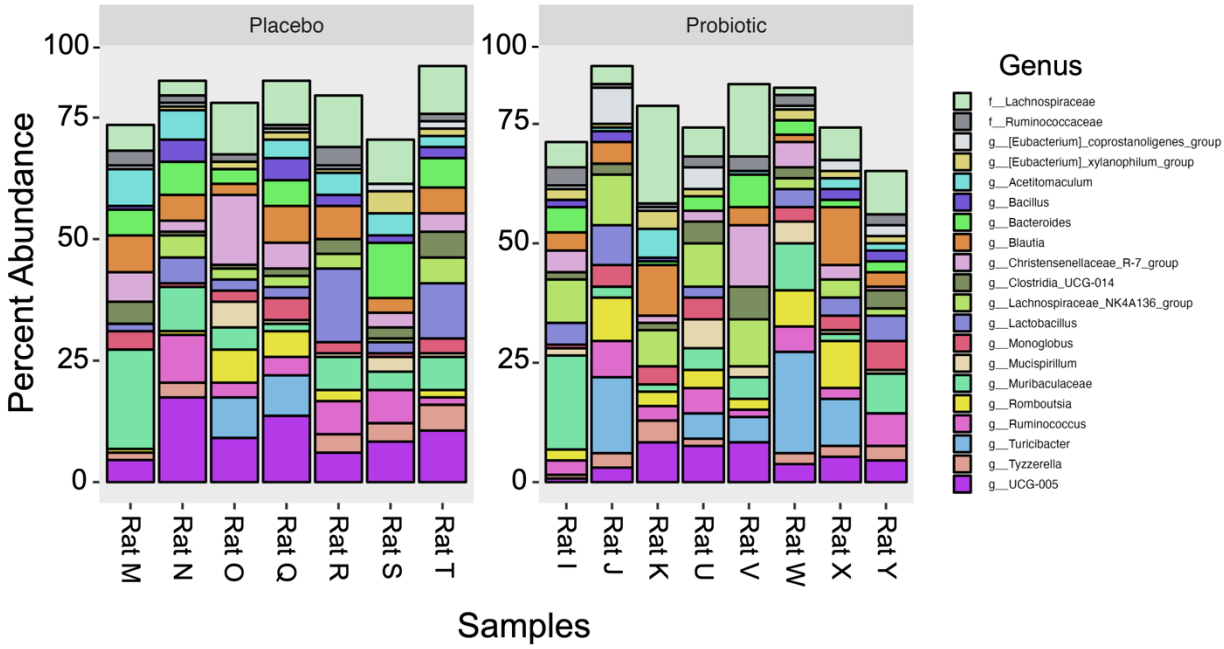

Figure 3.5 This figure shows the top six most abundant genera (L = 6) by treatment.

3.3.2.4 Differential Abundance

Differential abundance analyses were conducted using two separate methods: Microbiome Multivariable Association with Linear Models 2 (MaAslin2) and ANOVA-

Like Differential Expression tool 2 (ALDEx2). Due to differences amongst differential abundance analysis tools, Nearing and colleagues (2022) have recommended using multiple tools and employing a consensus approach. Using MaAslin2, 174 differentially abundant features were detected at a significance level of $q < .05$ (q-values indicate significance levels corrected using the Benjamini-Hochberg procedure). ALDEx2 detected 15 differentially abundant features at a significance level of $p < .05$, adjusted using a Holm-Bonferroni correction. Fifteen features were differentially abundant in both analyses and were considered positive results (see Table 3.2).

Table 3.2 Summary table of the fifteen differentially abundant features detected with MaAslin2 and ALDEx2 (Benjamini-Hochberg-corrected $q < .05$) with their respective effect sizes (MaAslin2 uses a regression coefficient and ALDEx2 uses standardized effect size) Study 1.

Feature	MaAslin2 (Effect Size)	ALDEx2 (Effect Size)
Muribaculaceae *Muribaculaceae* (ASV 615)	Increased in placebo rats (-0.24)	Increased in placebo rats (-4.77)
Tannerellaceae *Parabacteroides parabacteroides goldsteinii* (ASV 1105)	Increased in placebo rats (-0.08)	Increased in placebo rats (-3.56)
Lactobacillaceae *Lactobacillus lactobacillus intestinalis* (ASV 1331)	Increased in placebo rats (-0.15)	Increased in placebo rats (-3.26)
Unclassified Erysipelatoclostridiaceae *Erysipelotrichaceae UCG-003* (ASV 1071)	Increased in placebo rats (-0.08)	Increased in placebo rats (-3.40)
Lachnospiraceae *Tyzzerella* (ASV 1063)	Increased in placebo rats (-0.14)	Increased in placebo rats (-2.66)
Lachnospiraceae *Blautia* (ASV 1382)	Increased in placebo rats (-0.11)	Increased in placebo rats (-2.73)
Bacillaceae Bacillus *lactobacillus vaginalis* (ASV 81)	Increased in placebo rats (-0.12)	Increased in placebo rats (-2.98)
Erysipelatoclostridiaceae *Erysipelatoclostridium* (ASV 438)	Increased in placebo rats (-0.10)	Increased in placebo rats (-3.58)
Christensenellaceae *Christensenellaceae R-7 group* (ASV 695)	Increased in placebo rats (-0.19)	Increased in placebo rats (-3.82)
Unclassified Muribaculaceae *Muribaculaceae* (ASV 972)	Increased in placebo rats (-0.11)	Increased in placebo rats (-3.34)
Unclassfied Lachnospiraceae (ASV 1235)	Increased in placebo rats (-0.05)	Increased in placebo rats (-3.22)
Peptostreptococcaceae *Romboutsia* (ASV 1358)	Increased in placebo rats (-0.10)	Increased in placebo rats (-2.96)
Oscillospiraceae *NK4A214 group* (ASV 1364)	Increased in placebo rats (-0.06)	Increased in placebo rats (-3.17)
Deferribacteraceae *Mucispirillum Mucispirillum schaedleri* (ASV 584)	Increased in placebo rats (-0.11)	Increased in placebo rats (-2.53)
Unclassfied Lachnospiraceae (ASV 1136)	Increased in placebo rats (-0.06)	Increased in placebo rats (-2.50)

3.1.12 qPCR Strain Detection for Study 2

The qPCR strain detection results indicated that the probiotic strains were not detected in the placebo-treated animals when caecal contents were collected on the day of sacrifice (see Figure 3.6.A). However, the two probiotic strains used, *L. rhamnosus* R0011 and *L. helveticus* R0052, were detected in the probiotic-treated animals (see Figure 3.6.B).

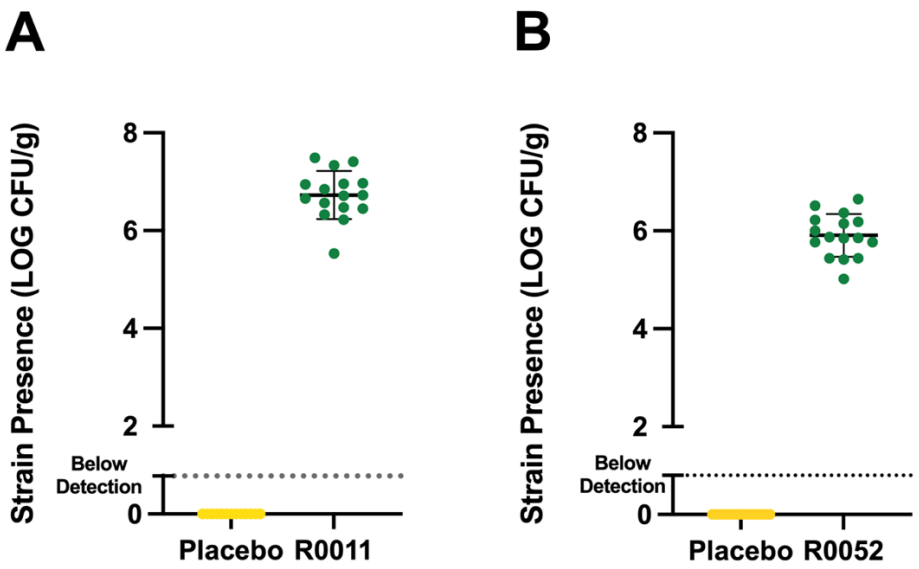

Figure 3.6 A) The strain presence of *L. rhamnosus* R0011, measured in LOG CFU per gram, in the animals' caecal content DNA, as determined by qPCR detection. B) The strain presence of *L. helveticus* R0052, measured in LOG CFU per gram, in the animals' caecal content DNA, as determined by qPCR detection.

3.1.13 Microbiota Analyses for Study 2

3.3.4.1 Species Richness (Alpha Diversity)

I tested for differences between Shannon, Simpson and Observed Features diversity indices using a factorial ANOVA (phyloseq package; McMurdie & Holmes, 2013). I found

no significant differences in the experimental groups' Shannon or Simpson indices. For Observed Features, a main effect of diet was revealed, such that the standard diet rats had a significantly higher number of Observed Features in their gut microbiota ($M = 94.19$, $SD = 9.88$) compared to their Western diet-fed counterparts ($M = 70.71$, $SD = 9.30$, $F_{(1, 26)} = 42.47$, $p < .001$, $\eta^2 = .99$; see Figure 3.7).

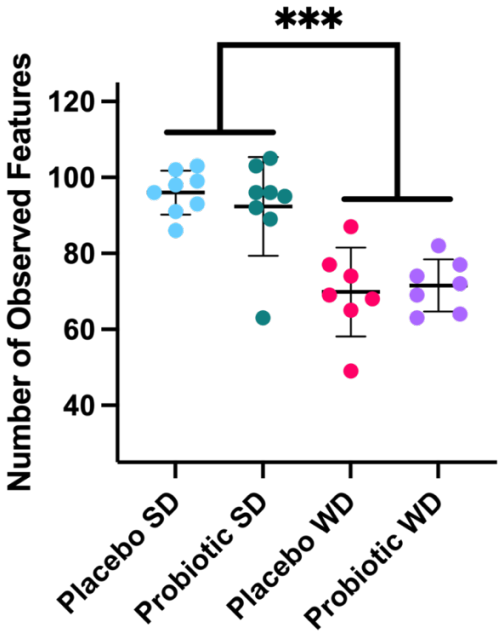

Figure 3.7 This figure illustrates the main effect of diet on the alpha diversity metric, Observed Features, in rat gut microbiota.

3.3.4.2 Species Diversity (Beta Diversity)

I used Weighted UniFrac, Unweighted UniFrac, Aitchison's Distance and PhILR metrics, analyzed with PERMANOVAs, to examine species diversity in the experimental groups. For Weighted UniFrac, a significant main effect of treatment ($F_{(1, 26)} = 6.12$, $p = .002$, $r^2 = .12$) and a significant main effect of diet were found ($F_{(1, 26)} = 17.53$, $p = .001$, $r^2 = .50$; see Figure 3.8).

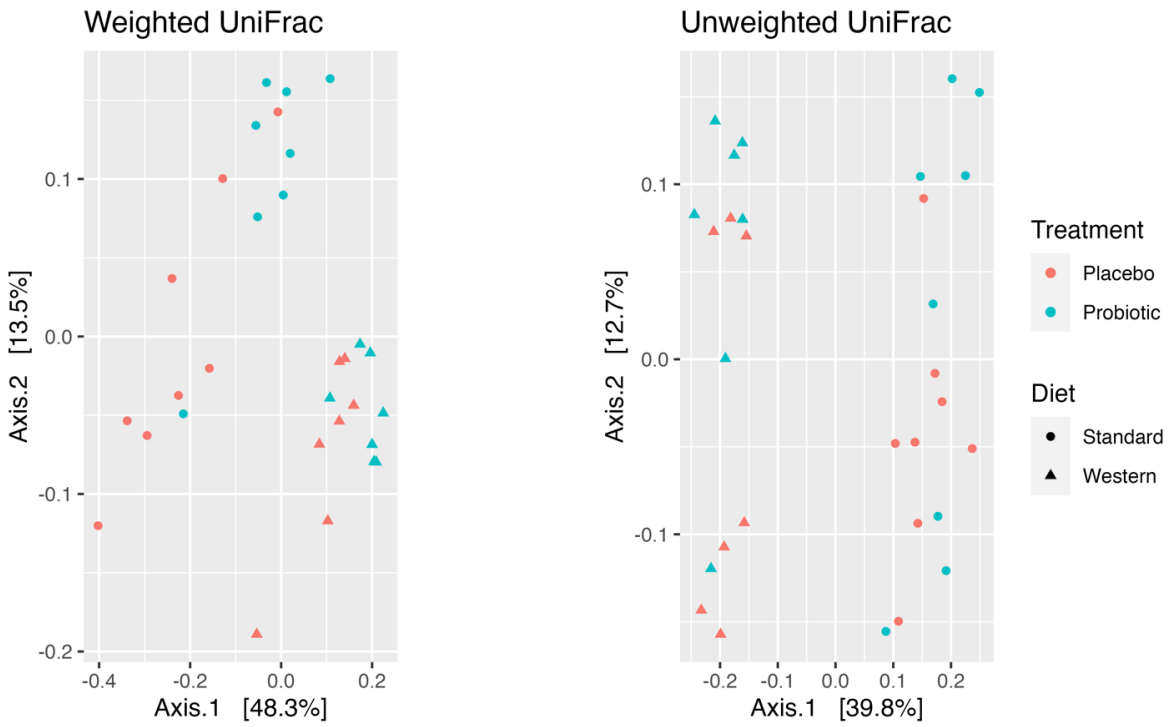

Figure 3.8 This figure illustrates the Weighted and Unweighted Unique Fraction (UniFrac) metrics of gut microbiota species diversity found in rats' caecal content DNA with data point colour indicating the animal's treatment group and data point shape representing diet group. To determine their differences, the Unique Fraction metric measures the fraction of phylogenetic tree branch lengths unique to each sample (Lozupone et al., 2011; Lozupone & Knight, 2005) Weighted UniFrac weights these differences based on the relative abundance of a feature sample (Lozupone et al., 2011; Lozupone & Knight, 2005), whereas Unweighted does not.

For Unweighted UniFrac, the main effects of treatment and diet were as follows: $F_{(1, 26)} = 3.40$, $p = .017$, $r^2 = .06$ for treatment and $F_{(1, 26)} = 22.09$, $p < .001$, $r^2 = .41$ for diet.

There was also a significant difference in Aitchson's Distance values based on diet administration ($F_{(1, 26)} = 15.67$, $p < .001$, $r^2 = .35$; see Figure 3.9). No interaction between diet and probiotic administration was found for Aitchison's Distance ($F_{(1, 26)} = 1.61$, $p = .091$, $r^2 = .04$).

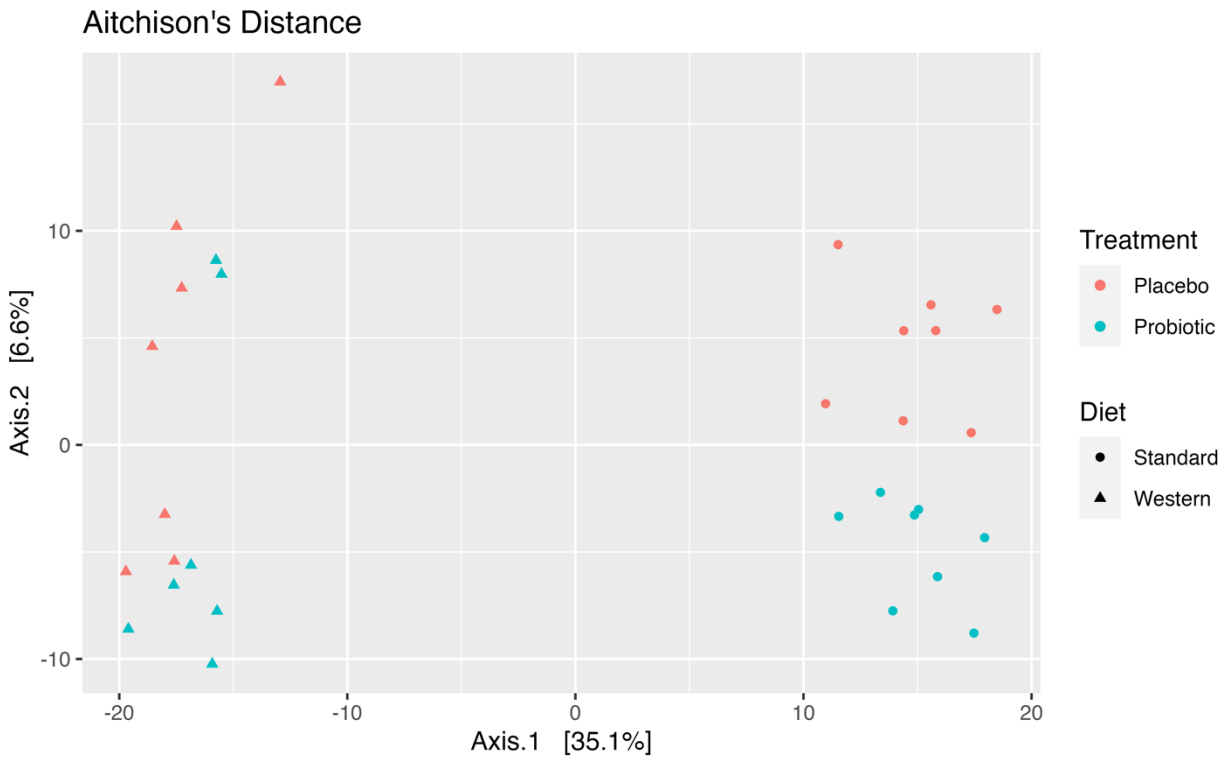

Figure 3.9 This figure illustrates the species diversity metric, Aitchison's Distance, showing the significant main effect of diet on this metric.

The main effects of both treatment and diet on the PhILR values were as follows: The PhILR values were significantly different between SD-fed and WD-fed dams ($F_{(1, 26)} = 15.17$, $p < .001$, $r^2 = .34$) as well as between the placebo- and probiotic-treated dams ($F_{(1, 26)} = 2.26$, $p = .046$, $r^2 = .05$; see Figure 3.10).

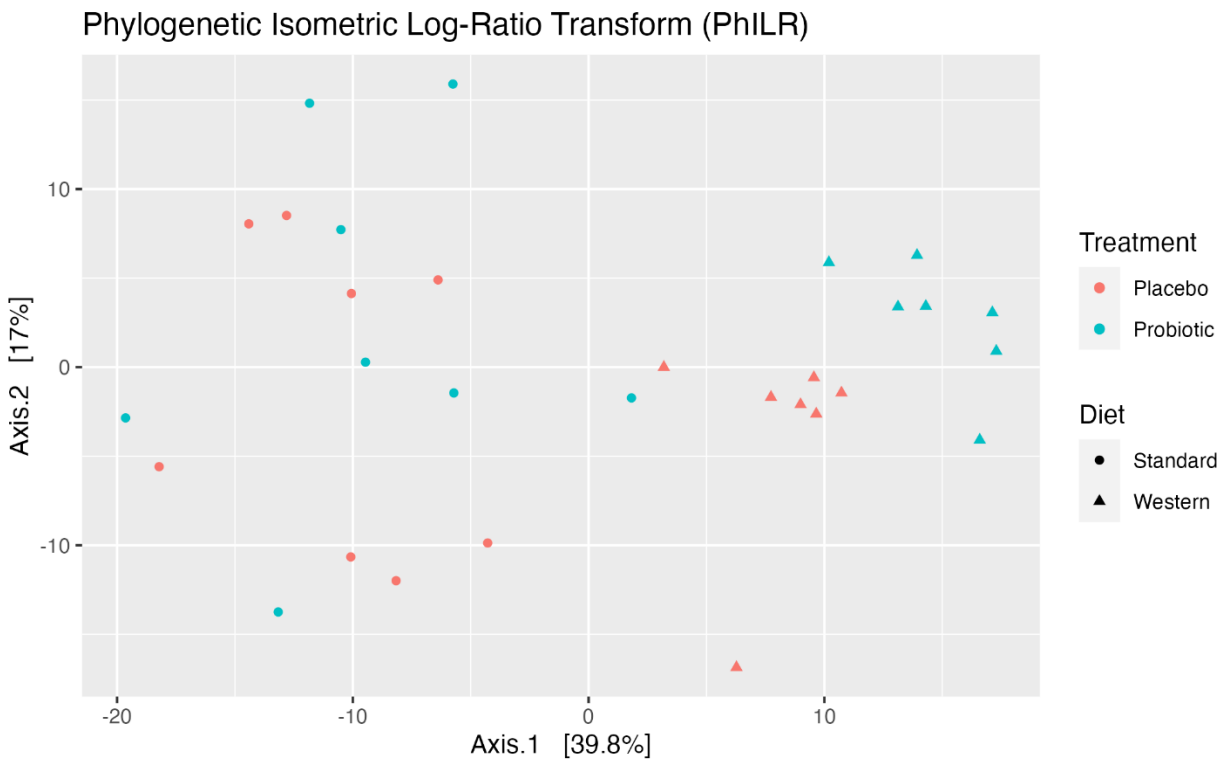

Figure 3.10 This figure illustrates the species diversity metric, PhILR, showing the significant main effects of diet and treatment on this metric.

3.3.4.3 Relative Abundance

Relative abundance of the top seven phyla (see Figure 3.11) and the top 15 genera (see Figure 3.12) is shown for each animal, grouped by treatment and diet. At the phylum level, I observed a general trend of higher Firmicutes and lower Bacteroidota in the placebo SD-fed animals and lower Firmicutes and higher Bacteroidota in the remaining groups. For

genus, I observed that there was a high level of variability between individual animals.

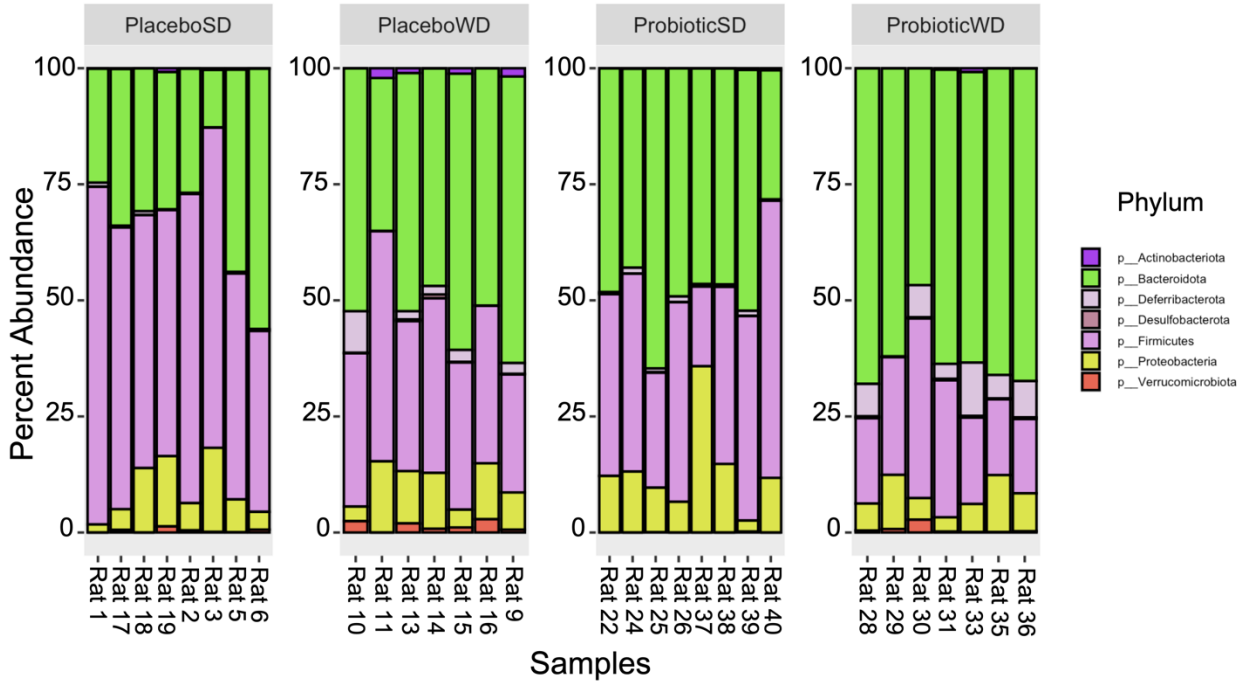

Figure 3.11 This figure shows the top seven most abundant phyla (L = 2) by treatment and diet groups.

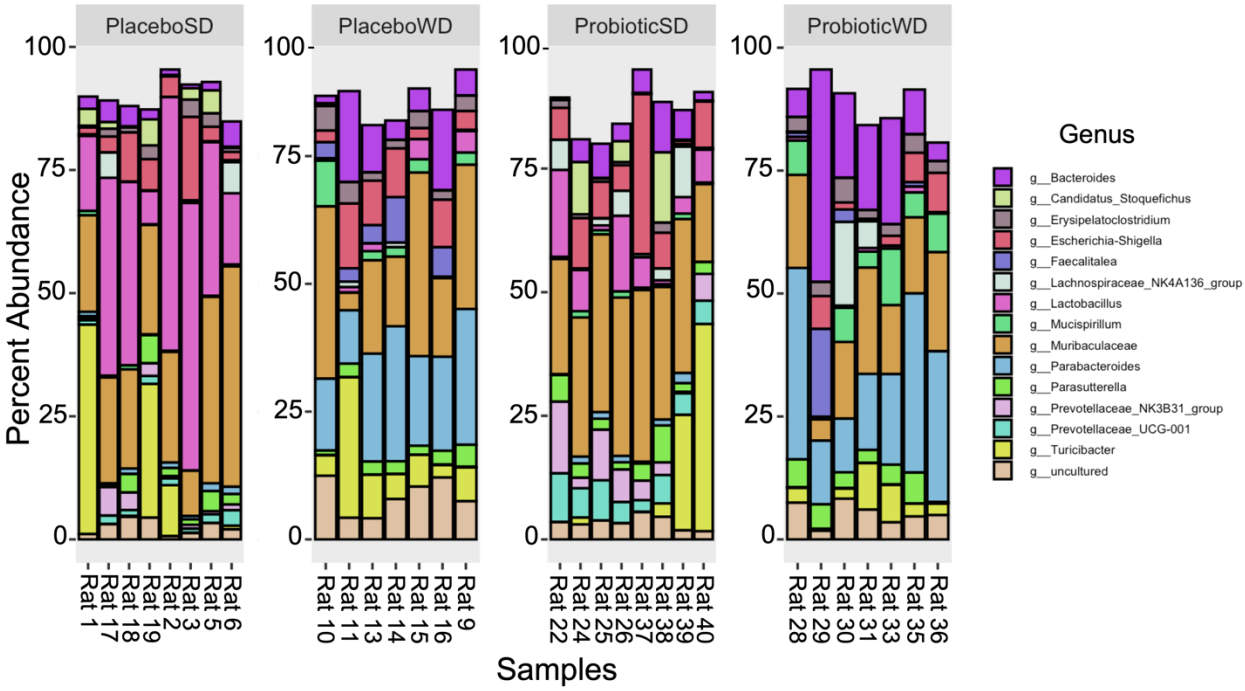

Figure 3.12 This figure shows the top 15 most abundant genera (L =6) by treatment and diet groups.

3.3.4.4 Differential Abundance

Study 2 investigated the differential abundance of features using two different analyses (MaAslin2 and ALDEx2) on relative abundance. MaAslin2 detected 38 differentially abundant features for diet, 12 for treatment, and six interactions between treatment and diet, all at a significance level of $q < .05$. ALDEx2 detected 13 differentially abundant features, all for diet, at a significance level of $p < .05$, adjusted using a Holm-Bonferroni correction. Thirteen features were common between the two analysis methods (see Table 3.3).

Table 3.3 Summary table of the thirteen differentially abundant features detected with MaAslin2 and ALDEx2 (Benjamini-Hochberg-corrected $q < .05$) with their respective effect sizes (MaAslin2 uses a regression coefficient and ALDEx2 uses standardized effect size) Study 2. WD = Western diet, SD = Standard diet.

Feature	MaAslin2 (Effect Size)	ALDEx2 (Effect Size)
Tannerellaceae *Parabacteroides parabacteroides goldsteinii* (ASV 51)	Increased in WD rats (0.40)	Increased in WD rats (2.93)
Unclassified Muribaculaceae *Muribaculaceae* (ASV 118)	Increased in SD rats (-0.18) Increased in probiotic SD rats (0.08)	Increased in SD rats (-2.93)
Unclassified Streptococcaceae *Lactococcus* (ASV 58)	Increased in WD rats (0.09)	Increased in WD rats (3.86)
Unclassified Muribaculaceae (ASV 177)	Increased in SD rats (-0.17) Increased in probiotic rats (0.07)	Increased in SD rats (-3.23)
Prevotellaceae *Prevotellaceae NK3B81 group* (ASV 221)	Increased in SD rats (-0.23) Increased in probiotic rats (0.11)	Increased in SD rats (-2.96)
Marinifilaceae *Odoribacter odoribacter laneus* (ASV 143)	Increased in WD rats (0.10)	Increased in WD rats (2.20)
Prevotellaceae *Prevotellaceae UCG-001* (ASV 148)	Increased in probiotic SD rats (0.10)	Increased in SD rats (-2.20)
Unclassified Muribaculaceae (ASV 26)	Increased in SD rats (-0.06)	Increased in SD rats (-1.90)
Unclassified Marinifilaceae *Odoribacter* (ASV 2)	Increased in WD rats (0.10)	Increased in WD rats (2.33)
Unclassified Muribaculaceae (ASV 36)	Increased in SD rats (-0.14)	Increased in SD rats (-2.25)
Lactobacillus intestinalis (ASV 247)	Increased in placebo rats (-0.08)	Increased in SD rats (-1.65)
Unclassified *Lactobacillus* (ASV 43)	Increased in SD rats (-0.20)	Increased in SD rats (-1.79)
Unclassified *Clostridium innocuum* (ASV 168)	Increased in placebo WD rats (0.11)	Increased in WD rats (1.28)

Discussion

Study 1 found that the probiotic strains used, *L. rhamnosus* R0011 and *L. helveticus* R0052 were detected only in the probiotic-treated animals. I found no differences based on probiotic treatment for alpha diversity. I found treatment differences in beta diversity for Unweighted UniFrac, Aitchison's Distance, and PhILR metrics.

At the phylum level, I saw no large differences in the relative abundance of bacteria between the treatment groups. I saw increased individual variability at the genus level but no large differences based on the treatment group. Lastly, 15 differentially abundant features were detected using MaAslin2 and ALDEx2.

Study 2 found that the probiotic strains (*L. rhamnosus* R0011 and *L. helveticus* R0052) were again detected only in the probiotic-treated animals. I found no significant differences in alpha diversity metrics based on treatment. I found that SD-fed dams had significantly higher observed features than WD-fed dams. For beta diversity metrics, a significant main effect of treatment was found for Weighted UniFrac, Unweighted UniFrac, and PhILR. I found main effects of diet on Weighted UniFrac, Unweighted UniFrac, Aitchison's Distance, and PhILR. For relative abundance, I observed higher Firmicutes in the placebo-treated SD-fed dams compared to other groups, followed by high individual variability at the genus level. Thirteen differentially abundant features were detected using MaAslin2 and ALDEx2.

3.1.14 Similarities between Study 1 and Study 2

3.4.1.1 Probiotic Strain Detection

In both studies within this chapter, the probiotic strains administered to the rats were detectable via qPCR but not via 16S rRNA sequencing. It is possible that these strains could be identified if the reference database used for microbial taxonomic assignment in these studies (SILVA) contained the strains' sequences. Evidence supports using custom databases specific to the organism and area of interest (e.g., bovine upper respiratory tract; (Myer et al., 2020). It has been found that there were fewer unassigned taxa when using a custom database containing longer sequence reads for bacteria within the bovine upper respiratory tract (Myer et al., 2020).

3.4.1.2 Alpha and Beta Diversity Metrics

Neither Study 1 nor 2 observed differences in alpha diversity metrics due to probiotic treatment; however, both studies had differences in two or more beta diversity metrics due to probiotic treatment.

There are conflicting results in the literature regarding how probiotics impact alpha diversity. In one study, male Wistar rats were given either a combined probiotic comprised of three different *Lactiplantibacillus plantarum* strains or a single *L. plantarum* strain (Q. He et al., 2021). Alpha diversity was increased in the rats receiving the combination probiotic (Q. He et al., 2021). In this study by He and colleagues (2021), the dosage was higher, 3×10^9 CFU per day for 14 days, compared to the 1×10^8 CFU provided to my rats for 6 weeks (Study 1) and 11 weeks (Study 2). It is possible that the increased CFU could be impacting alpha diversity or that the three probiotic strains, compared to the two strains used in this study, could be creating this difference. Multi-strain probiotic

treatments are theorized to boost efficacy (Ouwehand et al., 2018; Timmerman et al., 2004). It is also possible that these three strains of *L. plantarum* are metabolically complementary, based on recent research showing that one probiotic strain may complement another by providing it with the necessary nutrients for its proliferation (Khan et al., 2023). Conversely, in another rat study using male Wistar rats, researchers found no differences in alpha diversity in rats fed a multi-strain probiotic containing *Lactobacillus species* at a 1×10^8 CFU dose for 30 days (Sohail et al., 2017). This study by Sohail and colleagues (2017) is consistent with my findings in both Study 1 and Study 2, where probiotic administration, even with a multi-strain probiotic, does not significantly change alpha diversity. This is also consistent with findings from a study using healthy human participants where an unspecified multi-strain probiotic was provided, but alpha diversity was not significantly altered (Bagga et al., 2018). In Study 2, I observed that the number of features was indeed lower in the WD-fed dams, consistent with previous research showing that WD lowers alpha diversity (Y.-W. Wang et al., 2021) and even in studies where the animals are provided probiotics in addition to a WD, this does not result in improved alpha diversity (T. Wang et al., 2020; Q.-X. Zheng et al., 2021). Specifically, T. Wang and colleagues (2020) saw that in male C57BL/6J mice provided either standard or HFD (45% fat) and either *L. rhamnosus* LS-8 or *L. crustorum* MN047, that the HFD lowered alpha diversity, compared to SD animals, with no effect of either probiotic. Similar findings were observed in a study of pregnant female Sprague-Dawley rats examining WD administration combined with low and high doses of *L. rhamnosus* LGG and *Bifidobacterium* Bb12 (Q.-X. Zheng et al., 2021). In this study, compared to all of the WD-

fed groups, the control pregnancy group had the highest alpha diversity indices (Q.-X. Zheng et al., 2021).

My Study 1 and 2 results find that beta diversity is affected by probiotic treatment, as measured by a few different metrics. In Study 2, there are also differences in beta diversity based on diet. Although I did not find an interaction between probiotic administration and diet in Study 2, other researchers have found such differences. In a study where mice were given either an SD or a WD, *L. helveticus* R0052 (1×10^9 CFU) or a placebo, researchers found that the WD-fed mice without probiotic supplementation had the largest shift in microbiota composition (Ohland et al., 2013). In comparison, the mice fed WD along with *L. helveticus* R0052 supplementation closely resembled the placebo SD-fed group and the probiotic SD-fed group (Ohland et al., 2013). Another study examined female Sprague-Dawley rats on a WD for six weeks (Xu et al., 2023). One of the WD groups was also given 1×10^9 *Bacteroides vulgatus* Bv46 every other day (Xu et al., 2023). Researchers found that WD and probiotic administration affected beta diversity. WD animals clustered away from SD-fed animals, and the probiotic-treated WD animals significantly differed from the chow and WD-only groups (Xu et al., 2023). The findings from these studies are partially consistent with what I observed in both studies in this chapter. In Study 1, the dams were all on a standard chow diet, and the probiotic administration did not greatly shift the microbiota composition. In comparison, in Study 2, I observed that WD consumption did impact the gut microbiota, with fewer differences based on probiotic treatment. However, unlike Ohland and colleagues' (2013) findings, the probiotic treatment in Study 2 did not make the WD-fed animals' gut microbiome resemble that of the SD-fed controls. In the previously mentioned probiotic and WD study from T. Wang and colleagues (2020), they

also investigated beta diversity, finding that their mice clustered solely based on the type of diet they were fed (SD vs. HFD; T. Wang et al., 2020). These results are also consistent with the main effect of diet that I observed in my beta diversity analyses.

3.4.1.3 Relative Abundance

In Study 1, both the placebo- and probiotic-treated rats were fed the same SD given to rats in Study 2. In Study 1, I observed that both placebo- and probiotic-treated rats have a higher relative abundance of Firmicutes than the other detected phyla. Similarly, in Study 2, the placebo SD-fed rats have greater relative Firmicutes abundance than the other experimental groups. The other experimental groups have lower Firmicutes and increased Bacteroidetes. Both studies show high individual variability at the genus level. However, in Study 2, I observed that the placebo SD-fed dams have an increased relative abundance of *Lactobacillus* bacteria, and the WD-fed animals, regardless of their probiotic or placebo treatment, show increased levels of *Parabacteroides*. Like my findings, a study in male C57BL/6J mice saw that *Lactobacillus* bacteria were more abundant in the low-fat chow group (70% carbohydrates, 20% protein, 10% fat, 3.8 kcal/g) compared to the HFD group (B. Wang et al., 2020). Similar to my findings, a study of female Sprague-Dawley rats found that the HFD-fed probiotic-treated animals had increased Bacteroidetes and lowered Firmicutes compared to the chow-fed animals (Xu et al., 2023). It is hypothesized that the increase in Bacteroidetes may be attributed to an anti-obesity effect of the probiotic (Xu et al., 2023).

This study used male C57BL/6J mice and fed them an HFD (60% fat, 5.1 kcal/g) for 14 weeks (B. Wang et al., 2020). The gut microbiomes of both the control and HFD mice contained Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria, and found no

difference in the Firmicutes: Bacteroidetes ratio between these groups (B. Wang et al., 2020). Researchers found that HFD increased Firmicutes and decreased Bacteroidetes compared to the SD-fed animals, and HFD-fed animals demonstrated an increase in Proteobacteria compared to the SD-fed animals (T. Wang et al., 2020). Interestingly, this increase in Proteobacteria in the HFD-fed animals was reduced by probiotic administration of both *L. rhamnosus* LS-8 and *L. crustorum* MN047 (T. Wang et al., 2020) Firmicutes were more abundant in the GDM low- and high-dose probiotics groups than in normal pregnant dams (Q.-X. Zheng et al., 2021). However, similar to the present findings, a study of young and old (but otherwise healthy) male Sprague-Dawley rats found Firmicutes-dominant relative abundance in their gut microbiota (Lew et al., 2020). These conflicting results indicate that the Firmicutes to Bacteroidetes ratio may not be a good indicator of a healthy microbiome (Lew et al., 2020; Q.-X. Zheng et al., 2021).

3.4.1.4 Differential Abundance

The differential abundance analyses from Study 1 show that the probiotic treatment is not impacting the gut microbiome, as all 15 differentially abundant features detected by both MaAslin2 and ALDEx2 were increased in the placebo-treated animals. However, a few instances in Study 2 were found where probiotic treatment increased the abundance of a particular feature, as detected by MaAslin2. Notably, Study 2 had 15 animals each in the placebo and probiotic groups, so there were nearly double the animals in Study 1 when investigating treatment as a main effect. This increase in sample size may have facilitated detecting treatment effects. Despite the few differences in abundance based on probiotic treatment found in Study 2, the findings from both Studies 1 and 2 illustrate that probiotic treatment is only capable of subtle effects.

3.4.1.5 Specifics for Study 1 Differential Abundance Findings

In the placebo-treated animals, the genera *Muribaculaceae* was increased in abundance compared to probiotic-treated rats. An unclassified strain of *Muribaculaceae* was also increased in placebo-treated rats. *Muribaculaceae* is part of the bacterial family Muribaculaceae (formerly known as S24-7), which is commonly found in murines (Bowerman et al., 2021), including the blind mole rat, which is closely related to mice and has an abundance of Muribaculaceae in its gut (Sibai et al., 2020). Muribaculaceae have been shown to aid in producing propionate, a short-chain fatty acid (SCFA; B. J. Smith et al., 2019). SCFAs are important for the intestinal immune system (Postler & Ghosh, 2017) and correlate with increased longevity in mouse models (B. J. Smith et al., 2019). Researchers hypothesize that the blind mole rat's longevity may be due to an abundance of microbes, such as Muribaculaceae, that help produce SCFAs (Sibai et al., 2020).

I also found an increased abundance of *Parabacteroides goldsteinii* in my placebo-treated rats. However, there is conflicting evidence as to whether *Parabacteroides* bacteria are beneficial. Two studies using a specific *Parabacteroides goldsteinii* strain, MTS01, found positive results when providing it to their animals (Lai et al., 2022; Lin et al., 2022). The first study investigated MTS01 administration in a COPD mouse model, where researchers demonstrated that a specific *P. goldsteinii* MTS01 abundance was negatively correlated with the severity of chronic obstructive pulmonary disease (COPD) symptoms (Lai et al., 2022). *P. goldsteinii* MTS01 treatment also reduced proinflammatory cytokine levels in the colon (Lai et al., 2022). In a study using the same *P. goldsteinii* MTS01 strain in a mouse maternal immune activation model of autism, this probiotic treatment was provided to the offspring and reduced intestinal inflammation and autism-like behaviours

(Lin et al., 2022). In contrast, a study using a WD mouse model found that eight weeks of WD administration increased *Parabacteroides* and *Bacteroides*, with a decrease in known beneficial bacteria such as *Lactobacillus* and *Prevotella* (Everard et al., 2014). These results from Everard and colleagues (2014) are similar to mine. However, different *Parabacteroides goldsteinii* strains may lead to differing outcomes that depend on the experimental conditions.

Lastly, two genera from the family Erysipelotrichaceae (*Erysipelotrichaceae UCG-003* and *Erysipelatoclostridium*) are increased features in the placebo-treated dams. Mice treated with antibiotics and colorectal cancer patients also show increased levels of Erysipelotrichaceae (Kaakoush, 2015). There is evidence of increased Erysipelotrichaceae in inflammatory bowel disease (IBD) patients and animal models, but this evidence is inconsistent (Kaakoush, 2015). However, there is evidence from human studies that increased *Erysipelotrichaceae UCG-003* is associated with insulin resistance (Atzeni et al., 2022). Since these bacteria are only found in the placebo-treated animals, it is possible that the probiotic administered does not allow any Erysipelotrichaceae to flourish, despite my finding that the placebo-treated animals do have an abundance two *Lactobacillus* strains that demonstrate beneficial effects, *Lactobacillus intestinalis* (Lim et al., 2021; Q. Wang et al., 2023*)* and *Lactobacillus vaginalis* (Pan et al., 2019).

3.4.1.6 Specifics for Study 2 Differential Abundance Findings

Thirteen differentially abundant features were detected using MaAslin2 and ALDEx2. MaAslin2 detected main effects of treatment and diet, where an unclassified Muribaculaceae and *Prevotellaceae NKK3B81 group* were more abundant in SD-fed rats and probiotic-treated rats, whereas ALDEx2 only detected the increase in SD-fed rats.

Similarly, MaAslin2 detected an increase in *Prevotellaceae UCG-0001* in probiotic-treated SD-fed rats, where ALDEx2 found only a main effect of diet, such that this bacterium was increased in SD-fed rats. Both MaAslin2 and ALDEx2 found an increased abundance of *Lactobacillus* and two unclassified Muribaculaceae in SD-fed animals and an increased abundance of *Parabacteroides goldsteinii*, *Lactococcus*, *Odoribacter laneus* and unclassified *Odoribacter* in WD-fed animals.

Interestingly, the SD animals have an increased abundance of two different *Prevotellaceae* genera compared to the WD animals. This outcome makes sense within the context of previous literature, given that *Prevotellaceae* seems to grow well when higher dietary fibre is present (David et al., 2014; De Filippo et al., 2010). The fibre content of the SD used in Studies 1 and 2 is higher than that found in the WD.

I also found an increase of four different unclassified Muribaculaceae in the SD-fed animals, which is also consistent with a previous study where SD-fed C57BL/6J mice showed an increased abundance of unclassified Muribaculaceae compared to WD-fed animals (T. Wang et al., 2020). Interestingly, MaAslin2 detected an increase in one unclassified Muribaculaceae in SD-fed rats, but also probiotic-treated rats.

Contrary to my finding of increased *Lactobacillus* bacteria in SD-fed animals, T. Wang and colleagues found that in WD-fed mice supplemented with either *L. rhamnosus* LS-8 or *L. crustorum* MN047, the genus *Lactobacillus* was found to be differentially abundant compared to animals given WD only (T. Wang et al., 2020). I did not observe an increased differential abundance of *Lactobacillus* bacteria in either of the groups provided with *L. rhamnosus* R0011 and *L. helveticus* R0052.

I saw differences in the abundance of two *Prevotellaceae* strains. MaAslin2 detected an increase in *Prevotellaceae NK3B31* in SD-fed and probiotic-treated rats, separately. Only a difference in SD-fed rats was detected by ALDEx2. This finding is partially consistent with a study using male Sprague-Dawley rats on a WD; researchers found that supplementation of the WD with fermented soy paste increased certain bacteria compared to non-supplemented rats, including *Prevotellaceae* NK3B31 (Tung et al., 2020). Previous research in a Wistar rat model of alcohol use also shows that both *Prevotellaceae* NK3B31 and *Prevotellaceae* UCG-001 are decreased in the alcohol-dependent group (F. Yang et al., 2021), suggesting that *Prevotellaceae* bacteria only grow in beneficial environments.

I also saw that *Prevotellaceae* UCG-001 were increased in probiotic-treated, SD-fed rats (MaAslin2) and SD-fed rats (ALDEx2). In previous research in genetically obese (*ob/ob*) mice, the administration of inulin, a prebiotic fibre, resulted in an increased abundance of *Prevotellaceae* UCG-001 (X. Song et al., 2019). *Prevotellaceae* UCG-001 was then positively associated with the AMPK signalling pathway, which helps regulate glycolipid metabolism (X. Song et al., 2019). The decreased relative abundance of *Prevotellaceae* UCG-001 was found in a constipated rat model compared to their healthy control counterparts (L. Yang et al., 2022). Therefore, it is understandable that *Prevotellaceae* UCG-001 would be increased in the SD-fed rats, as they receive more fibre from their diet than their WD-fed counterparts.

Lastly, similar to the findings regarding *Parabacteroides goldsteinii*, there are mixed results on *Odoribacter*. In one study, *Odoribacter lanes,* when used as a probiotic, improved glucose tolerance in diet-induced obese mice (Huber-Ruano et al., 2022).

However, more broadly, the genus *Odoribacter* has also been shown to increase in abundance in WD-fed mice (Liang et al., 2021; Y. Liu et al., 2019).

3.4.1.7 Overlap in Differential Abundance Findings

There was some overlap in the differentially abundant bacteria amongst the experimental groups between Study 1 and 2. Both studies found increases in *Lactobacillus intestinalis;* Study 1 found these bacteria increased in placebo-treated rats, and Study 2 found this increase in placebo-treated or standard diet rats, according to MaAslin2 and ALDEx2, respectively. These findings demonstrate that rats possess endogenous *Lactobacillus* spp. consistent with other studies (D. Li et al., 2017; Yajima et al., 2001). Both studies also showed an increased abundance of unclassified bacteria from the family Muribaculaceae. This increased abundance of Muribaculaceae was observed in placebo-treated rats from Study 1 and standard diet-fed rats in Study 2. Interestingly, one of the Muribaculaceae *sp*. was detected as increased in both SD and WD-fed animals provided the probiotic in Study 2 (by MaAslin2). These findings of increased Muribaculaceae across all groups except for the placebo-treated WD-fed only animals are broadly lined up with the literature, suggesting that these bacteria are generally positively associated with longevity and a normal healthy diet in rodent models (Sibai et al., 2020). Lastly, differences were observed in both Study 1 and Study 2 concerning the abundance of *Parabacteroides goldsteinii*. Study 1 found these bacteria increased in placebo rats, whereas Study 2 detected an increase in WD-fed rats. These contradictory findings also coincide well with the mixed research on *Parabacteroides goldsteinii,* as previously mentioned, with some studies reporting the genus *Parabacteroides* to be associated with a poorer microbiome

(Everard et al., 2014) and some studies demonstrating the probiotic potential of a specific *Parabacteroides goldsteinii* strain (Lai et al., 2022; Lin et al., 2022).

Based on these findings, WD administration may shift the gut microbiota to not necessarily harmful microbes but towards a microbial profile beneficial only in some environments (e.g., *Parabacteroides, Odoribacter*). Meanwhile, the SD promotes more traditionally beneficial microbes such as *Lactobacillus* and Muribaculaceae. Again, there is a hint that the probiotic may help bolster this beneficial environment by increasing the abundance of unclassified Muribaculaceae and *Prevotellaceae* UCG-001. Still, the probiotic does not cause large shifts in microbiome composition.

3.1.15 Limitations and Future Directions

There are several limitations in the present studies to consider. First, I have no non-pregnant females who were given diet and treatment that I could compare to the pregnant females. In the future, this could be a good comparison group to parse out further differences specific to pregnancy. For example, many comparisons to prior research are being made against male rodents, which could significantly differ in some gut microbiota alterations. The use of only male rodents is a limitation commonly found in animal models across many disciplines, including neuroscience, general biology and pharmacology (Beery, 2018; Beery & Zucker, 2011). Furthermore, there are some suggestions in bioinformatics literature suggesting that *in vivo* studies should increase sample sizes (R. J. Moore & Stanley, 2016). In both studies included in this chapter, sample sizes are only seven or eight rats per group. In studies of rat microbiota, there are usually 6-12 animals per group (Čoklo et al., 2020) which is typical for disease models (R. J. Moore & Stanley,

2016) or investigating the effects of diet on behaviour (Korgan et al., 2018; Myles et al., 2023). Additionally, the data could have been influenced by cage effects, such that cage mates' gut microbiomes are more like one another than others in the same experimental designation (Alexander et al., 2006). Previous work also shows that different mouse strains have different gut microbiome compositions, illustrating that genetic differences between strains play a role in the microbiome (Alexander et al., 2006).

In future studies, analyzing the metabolome in addition to the microbiome may provide a more in-depth insight into what the diet and probiotic administration are doing. Diet is a massive factor in gut microbial composition (R. J. Moore & Stanley, 2016). Still, most studies have not focused on how specific dietary components might influence the gut microbiome during pregnancy (Barrientos et al., 2024). Beyond probiotics, other nutritional supplements, such as polyphenols (Masumoto et al., 2016; Rodríguez-Daza et al., 2020) and tannins (W. Zhu et al., 2018), can impact gut microbiota composition, too. A diet that includes polysaccharides and polyphenols is important for the growth of beneficial bacteria such as *Bifidobacterium*, *Lactobacillus* and *Prevotellaceae* genera (Larsen et al., 2019; Mao et al., 2019). Adding polyphenols and polysaccharides to a high-fat, high-sugar diet was sufficient to shift the gut microbiome composition of these mice to a Prevotellaceae and Akkermansiaceae enterotype (Rodríguez-Daza et al., 2020). This enterotype was like the standard chow diet animals (Rodríguez-Daza et al., 2020). Looking at the species diversity between the high-fat, high-sugar animals with and without adding polyphenols showed differences in the microbiota species diversity between these two groups (Rodríguez-Daza et al., 2020). Mice provided with apple procyanidins as a health supplement have shown an increase in the Muribaculaceae bacterial family (Masumoto et

al., 2016). Another study, which provided a persimmon tannin supplement to male Sprague-Dawley rats, showed that this supplement increased *Bifidobacterium* and *Lactobacillus* genera and decreased *Escherichia coli* and *Enterococcus* (W. Zhu et al., 2018). Future studies may want to investigate the similarities between probiotic and polyphenol supplementation and whether both interventions are recruiting similar mechanisms of action.

Lastly, longitudinal studies with fecal samples may be beneficial in capturing temporal changes in the microbiome composition (Quince et al., 2017) that occur due to diet and probiotic administration.

CHAPTER 4 THE EFFECTS OF MATERNAL DIET AND PROBIOTIC ADMINISTRATION ON OFFSPRING STRESS AND METABOLIC MARKERS

INTRODUCTION

Research into the developmental origins of health and disease (DOHaD) began with investigating how the Dutch famine (November 1944 to May 1945) affected adult health outcomes of people exposed *in utero* to this early-life nutritional deprivation (D. J. P. Barker & Osmond, 1986). In the 1986 study by Barker and Osmond, it was found that early-life malnutrition and low birth weight were linked with an increased risk of adult death due to cardiac disease. Since this study, the importance of events during early development, especially during the first 1000 days of life (i.e., from conception to two years of age), has been increasingly researched (Enos et al., 2013; Roseboom et al., 2001) with both the depth and breadth of topics being expanded. Today, DOHaD research investigates all life stages and looks at factors ranging from chemical exposure to mental and physical stressors (as summarized by Suzuki, 2018).

4.1.1 Maternal High-Fat/Western Diets as Developmental Stressors

A diet high in fat (e.g., a high-fat diet [HFD] or a Western diet [WD]) consumed during gestation is a physiological stressor (Bellisario et al., 2014; Reynolds et al., 2013). Thus, it isn't surprising that WD consumption in pregnant rats can impact the development of the primary stress response system, the hypothalamic-adrenal-pituitary (HPA) axis, in offspring exposed to this diet *in utero* (Legendre & Harris, 2006). There are similarities in offspring neuroendocrine profiles from mothers who were given WD (Bellisario et al.,

2014) compared to those exposed to stress during gestation (Louvart et al., 2009). Specifically, Bellisario and colleagues (2014) found that adult offspring (including males and females) from wild-type mice (C57BL6/J background) fed a WD throughout gestation had increased corticosterone levels in response to an acute stressor compared to offspring from dams fed a control diet. Similarly, Louvart and colleagues (2009) found that adult female Sprague-Dawley rats from prenatally stressed dams had increased basal corticosterone levels post-foot shock stressor. This sensitization of the HPA axis (i.e., increased corticosterone levels) in offspring exposed to WD *in utero* can occur due to increased exposure to corticosterone. During pregnancy, corticosterone levels in pregnant rodents are five to ten times higher in maternal plasma compared to the fetus (Dalle et al., 1978 as cited by Bellisario et al., 2015; Montano et al., 1991). Therefore, to protect developing rodent offspring from exposure to excess glucocorticoids, the placental enzyme 11-beta-hydroxysteroid dehydrogenase type 2 (11β-HSD-2) converts active corticosterone into its inert form, 11-dehydrocorticosterone (Bellisario et al., 2015). Specifically, this lower comparative corticosterone in fetuses than in dams is likely the result of this placental 11β-HSD-2 enzyme activity (R. W. Brown et al., 1996). However, importantly, studies demonstrate that maternal HFD lowers 11β-HSD-2 activity in the placental tissue, which can result in HPA axis sensitization in adult offspring (Bellisario et al., 2015).

Both HFD and WD are similar in that they include nutritional elements that are processed, high-fat and low-fibre (Christ et al., 2019; Imamura et al., 2015; Monteiro et al., 2013). Therefore, these types of diets can increase the risk of offspring developing metabolic disorders (Samuelsson et al., 2008) For example, maternal obesity and type 2 diabetes (T2D) are strong predictors of childhood obesity (Rooney et al., 2011; Werneck

et al., 2017). Further, children from mothers who had gestational hypertension often have higher body mass indices (BMIs), higher blood pressure and increased insulin levels (Cho et al., 2000). In overweight or obese mothers, there is also an increased risk of fetal macrosomia (i.e., a fetus weighing more than nine to ten pounds), which can increase the risk of metabolic syndrome in children (Dunlop et al., 2015). Metabolic syndrome is defined as a person with three out of five abnormal measurements from elevated triglycerides, elevated blood pressure, reduced high-density lipoprotein cholesterol, elevated fasting glucose, or elevated waist circumference (Nilsson et al., 2019). Similar changes in rat offspring birth weight following maternal WD or HFD administration have been observed, although findings can differ from study to study. For instance, a study of pregnant Sprague-Dawley rats provided either a standard diet (SD) or an HFD (60% kcal from fat; D12492, Research Diets, Inc. USA) throughout gestation (3 weeks) found no difference in offspring birth weights (Purcell et al., 2011). Conversely, another study in pregnant Sprague-Dawley rats given an HFD for a prolonged period (14 weeks; 45% kcal from fat; D12451, Research Diets, Inc. USA) or a control diet (10% kcal from fat, D12450B) saw that offspring birth weight was increased (Y. Song et al., 2015). In a meta-analysis on women with gestational diabetes mellitus (GDM), there was a tendency for infant birth weight to be lower, as compared to study controls (C.-C. Wang et al., 2020).

It is well-accepted that breast milk hormones, such as the adipokines leptin and insulin, are crucial for offspring metabolic development (Schuster et al., 2011). Adipokines such as leptin and insulin are secreted from adipose tissue and are involved in metabolic processes (Berg & Scherer, 2005; Ouchi et al., 2003). For instance, leptin can regulate food intake and energy use by binding to leptin receptors (Ob-r), particularly in the arcuate

nucleus of the hypothalamus (Zepf et al., 2016). It has also been repeatedly reported that a relationship exists between high plasma leptin and adiposity in both children and adults (Caprio et al., 1996; Considine et al., 1996; Garcia-Mayor et al., 1997; Havel, 1998; Nagy et al., 1997). Importantly, leptin is transferrable to breast milk from maternal blood (Casabiell et al., 1997), and maternal serum leptin and breast milk leptin levels have been positively correlated (Kratzsch et al., 2018). However, the amount of breast milk leptin derived from maternal plasma remains unknown compared to how much leptin is synthesized directly in the mammary glands (Palou et al., 2018). Higher leptin levels are associated with obesity, and obese rat dams have higher leptin content in breast milk (Castro et al., 2017). It is presently unknown if there is an "optimal" amount of leptin to provide to offspring, so their development and growth are normal (Bautista et al., 2008; Palou et al., 2018; Sánchez et al., 2005). Previous research has also found a positive correlation between maternal BMI and milk leptin; researchers postulate that offspring from overweight mothers are likely exposed to more significant amounts of leptin via milk consumption (Kratzsch et al., 2018). A systematic review investigating the correlation between maternal BMI and gut hormones found ten studies where breast milk leptin levels positively correlated with maternal BMI (Andreas et al., 2014). Further demonstrating the connection between maternal weight and offspring metabolic programming, breastfed infants' serum leptin levels significantly and positively correlated with maternal BMI (Savino et al., 2006). In contrast, this correlation is not observed in formula-fed infants (Savino et al., 2006).

Along with leptin, insulin can be investigated in breast milk. Insulin is a peptide hormone produced by pancreatic β-cells, released into the bloodstream in response to

nutrient intake to aid in glucose homeostasis (J. Lee & Pilch, 1994; Petersen & Shulman, 2018). Many cell types have insulin receptors, although insulin's direct effects on skeletal muscle, liver and adipose tissue are most important for its homeostatic role (J. Lee & Pilch, 1994; Petersen & Shulman, 2018). Insulin is also present in the breast milk of both humans (Shehadeh et al., 2001) and rats (Buts et al., 1997). Similar to leptin, insulin is positively correlated with maternal BMI in humans (Andreas et al., 2014; Chan et al., 2018; Young et al., 2017). In the 2014 review by Andreas and colleagues, they reported on four studies investigating breast milk insulin levels. Two of these studies reported a positive correlation between maternal BMI and breast milk insulin; the others reported no correlation (Andreas et al., 2014). A study of Canadian women showed that pre-pregnancy BMI was positively correlated with maternal milk insulin (Chan et al., 2018). Another breastfeeding study in American women demonstrated that maternal plasma insulin was positively correlated with breast milk insulin, and both insulin measures were positively correlated with maternal BMI at 2 weeks and four months postpartum (Young et al., 2017).

4.1.2 Maternal Diet and Breast Milk

There is limited research on how diet composition affects the hormone profiles of breast milk. Two studies working with rat models of maternal HFD found no difference in milk leptin levels between their experimental and control groups (Gorski et al., 2006; Purcell et al., 2011). In humans, it has been reported that there is no correlation between maternal diet and the nutrient composition of breast milk (as reviewed by Łubiech & Twarużek, 2020). Still, it appears that fatty acids (e.g., docosahexaenoic acid; DHA, eicosapentaenoic acid; EPA; α-linoleic acid; ALA) in breast milk are impacted by maternal diet. A

systematic review by Keikha and colleagues (2017) found a small inverse association between the sugar content of breast milk and maternal adiposity (as measured by body fat percentage). Additionally, similar to Łubiech and Twaruzek (2020), this systematic review included studies showing that diets with increased fatty acids (e.g., DHA, EPA) and micronutrients (e.g., vitamin B1, vitamin B12) are related to the mothers' diet (Keikha et al., 2017). For example, one study found that lower protein intake was associated with increased DHA levels in breast milk (Tiangson et al., 2003). Another study found that maternal diets higher in vitamin B1 were positively correlated with B1 levels in breast milk (Ortega et al., 2004). Therefore, it is possible that hormones, like fatty acids, are influenced by diet composition.

4.1.3 Does Maternal Diet or Probiotic Treatment Impact Milk Hormones?

Due to the critical programming effects of maternal milk hormones such as leptin and insulin on offspring metabolism, the first objective of this work was to explore how maternal diet affects leptin and insulin levels in breast milk. A second objective was to examine whether probiotic treatment in dams affects milk hormone levels given that some indications are that probiotic treatment may modulate breast milk composition (Łubiech & Twarużek, 2020). However, there is also conflicting evidence on whether probiotic treatment affects leptin levels in breast milk. One study found that a multi-strain probiotic reduced plasma leptin levels in male rats fed a high-fat, high-sucrose diet for 16 weeks (Al-muzafar & Amin, 2017). Another study found neither single-strain nor multi-strain probiotics could reduce elevated leptin levels in genetically obese male Zucker-Lepr$^{fa/fa}$ rats (Plaza-Diaz et al., 2014).

4.1.4 Maternal Care Programs the Offspring HPA Axis

As discussed in Chapter 1, rodent maternal care behaviour is crucial for programming the offspring's stress response (Weaver et al., 2004). When dams engage in high quantities of licking/grooming (LG) and arched-back nursing (ABN) behaviours, this results in increased glucocorticoid receptor (GR) expression in the offspring's hippocampi (Weaver et al., 2004). Specifically, in Long-Evans rats, offspring exposed to increased amounts of LG-ABN behaviour seldom had methylation at the exon 1_7 promoter sequence of the GR gene, resulting in increased expression of GR (Weaver et al., 2004). This increase in GR expression makes offspring more sensitive to negative feedback when glucocorticoids bind to these receptors (Weaver et al., 2004), helping to shut down HPA axis activity promptly (De Kloet et al., 1998). Thus, anything that affects maternal care, including diet or probiotic treatment, many also impact the programming of offspring stress responding.

Maternal nutrition contributes to fetal growth and development and health and disease in postnatal life; therefore, the mitigation of future disease risk in offspring is an area requiring further research. Compared to maternal diet, there is less evidence of how probiotic treatment affects maternal care and subsequent offspring outcomes. However, there is preliminary evidence that maternal probiotic administration of Lacidofil® strains is beneficial for maternal care behaviours. Specifically, treatment with Lacidofil® has been reported to increase the frequency of active maternal care behaviours such as LG and ABN, compared to placebo administration (O'Leary, 2019). Thus, based on preliminary research, probiotic treatment is an area of interest for counteracting the effects of stressors, potentially via modulating maternal care in addition to other means.

4.1.5 Probiotic Treatment and Physiological Stressors

As previously discussed, maternal WD or HFD and prenatal stress exposure can affect the offspring's stress response development. Relatedly, probiotics have several different functions that are valuable to study for possible clinical relevance in perinatal health for mothers and their offspring (Anukam et al., 2006; Brantsaeter et al., 2011; Monachese et al., 2012; Yeganegi et al., 2009). To date, the probiotic Lacidofil® has been studied for its role in mitigating the effects of acute stressors in infant offspring, when the probiotic itself was provided to nursing dams (Callaghan & Tottenham, 2016; Cowan et al., 2016; Cowan & Richardson, 2019) and regulating HPA axis activity after stress exposure, when given directly to rats experiencing the stressor (Gareau et al., 2007; Natale et al., 2021). One study demonstrated Lacidofil®'s ability to improve rats' stress resilience in a chronic, unpredictable stress paradigm (Natale et al., 2021). In addition to Lacidofil® increasing stressed rats' exploratory behaviours in an open-field test and decreasing their floating behaviour across trials in a swimming task, the probiotic-treated rats had higher dehydroepiandrosterone (DHEA) to corticosterone ratios, indicative of lessened stress responsivity (Natale et al., 2021). Lacidofil® has also been shown to normalize basal corticosterone levels in rat offspring exposed to maternal separation stress (Gareau et al., 2007).

Previous animal studies have shown that the gut microbiome is altered at puberty, but the microbiome's composition also affects sex hormone levels (Markle et al., 2013). For example, *Lactobacillus* strains (such as those found in Lacidofil®) are known to secrete β-glucuronidase, an enzyme that helps active estrogen via deconjugation (Plottel & Blaser, 2011). Since probiotic administration mitigates some of the effects of early-life stress on

microbial composition, there may also be a place for probiotic treatment to mitigate other effects of early-life stress (Cowan & Richardson, 2019). In a study by Cowan & Richardson (2019), early life stress through maternal separation resulted in accelerated female puberty onset and delayed male puberty onset. One group of dams received Lacidofil® in their drinking water, and this group's offspring (both female and male) did not have pubertal timing disruptions (Cowan & Richardson, 2019). These results add to the literature surrounding the benefits of the probiotic Lacidofil® in situations of early life stress and allude to the possibility that the gut microbiome may influence sex hormone levels (Cowan & Richardson, 2019). Due to these findings, I also investigated whether maternal Lacidofil® administration altered anogenital distance at birth if provided throughout pregnancy.

4.1.6 Objectives and Hypotheses

Overall, this chapter explores the impact of administering the probiotic Lacidofil® (*Lacticaseibacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052) to pregnant female rats and examines outcomes related to offspring health.

Study 1 investigated the effects of maternal probiotic administration on offspring birth weight and anogenital distance. Further, the metabolic hormones insulin and leptin were analyzed in breast milk samples. Study 2 investigated whether prolonged WD and probiotic administration in rat dams impacts offspring outcomes, including stress responding (i.e., hippocampal glucocorticoid receptor expression, plasma corticosterone levels) and metabolic variables (i.e., plasma leptin levels). Additionally, leptin levels in breast milk were measured, and offspring birth weights were reported.

4.1.6.1 Study 1

No hypotheses were made about how maternal probiotic treatment would affect offspring birth weights, anogenital distances or milk hormone levels, as previous work with maternal administration of the Lacidofil® probiotic did not report differences in offspring birth weights (Callaghan et al., 2016; Cowan et al., 2016) or newborn anogenital distances (Cowan & Richardson, 2019) based on treatment. To my knowledge, this is the first study investigating the effects of Lacidofil® on breast milk hormones in rats.

4.1.6.2 Study 2

Based on the findings regarding probiotics increasing active maternal care (O'Leary, 2019), it was first hypothesized for this study that offspring from WD-fed, probiotic-treated dams would have increased hippocampal glucocorticoid receptor expression compared to the offspring from WD-fed, placebo-treated dams. Second, it was hypothesized that offspring from WD-fed and placebo-treated dams would have higher basal levels of plasma corticosterone compared to offspring from SD-fed and probiotic counterparts, respectively. I hypothesized that offspring from probiotic-treated, WD-fed dams would have lower plasma corticosterone than those from placebo-treated, WD-fed dams. Third, it was hypothesized that the offspring of WD-fed dams would have higher plasma leptin levels than those of SD-fed dams. No specific hypotheses were made about whether maternal probiotic administration would impact offspring plasma leptin levels. Fourth, it was hypothesized that WD-fed dams would have higher leptin levels in their breast milk than their SD-fed counterparts. Again, there were no specific hypotheses about whether maternal probiotic administration would affect breast milk leptin levels.

It was hypothesized that maternal WD administration would increase offspring birth weights. No hypotheses were made about how maternal probiotic administration would affect offspring birth weight or how it would interact with the maternal diet. Offspring anogenital distances were not measured in Study 2.

METHODS

4.1.7 Study 1: Animals and Breeding

Male (225-250 grams; n = 10) and female (200-225 grams; n = 20) Long-Evans rats were obtained from Charles River Laboratories (St. Constant, QC, Canada) for this experiment. Animals were pair-housed in same-sex pairs and quarantined for 14 days before the experiment began in polypropylene cages (47 cm x 24 cm x 20.5 cm) with wire lids. The cages contained a softwood bedding blend (Fresh Bed, Shaw Resources, Shubenacadie, NS, Canada) and one black polyvinyl chloride (PVC) tube measuring 12 cm long x 9 cm in diameter for enrichment. During quarantine and the experiment, colony rooms were maintained at 20°C $\pm$ 2°C with a reverse light cycle, with lights off from 1000h to 2200h. All rats were provided Laboratory Rodent Diet 5001 (LabDiet®, St. Louis, MO, United States of America) and tap water *ad libitum*. All procedures performed during this experiment were done under the guidance of the Canadian Council on Animal Care (CCAC) and were approved by the Dalhousie University Committee on Laboratory Animals (UCLA) under ethics protocol approval #19-028.

After the quarantine period, animals were randomly and equally separated into two separate colony rooms, one designated for the placebo group and one designated for the probiotic group, to minimize cross-contamination risk. Breeding for this experiment was

conducted in two identical phases. One male and one naïve female rat were paired for seven consecutive days. After this breeding period, males and females were separated, and females were housed alone for the experiment; this day was considered gestational day (GD) 0 (see Figure 4.1 for the experimental timeline).

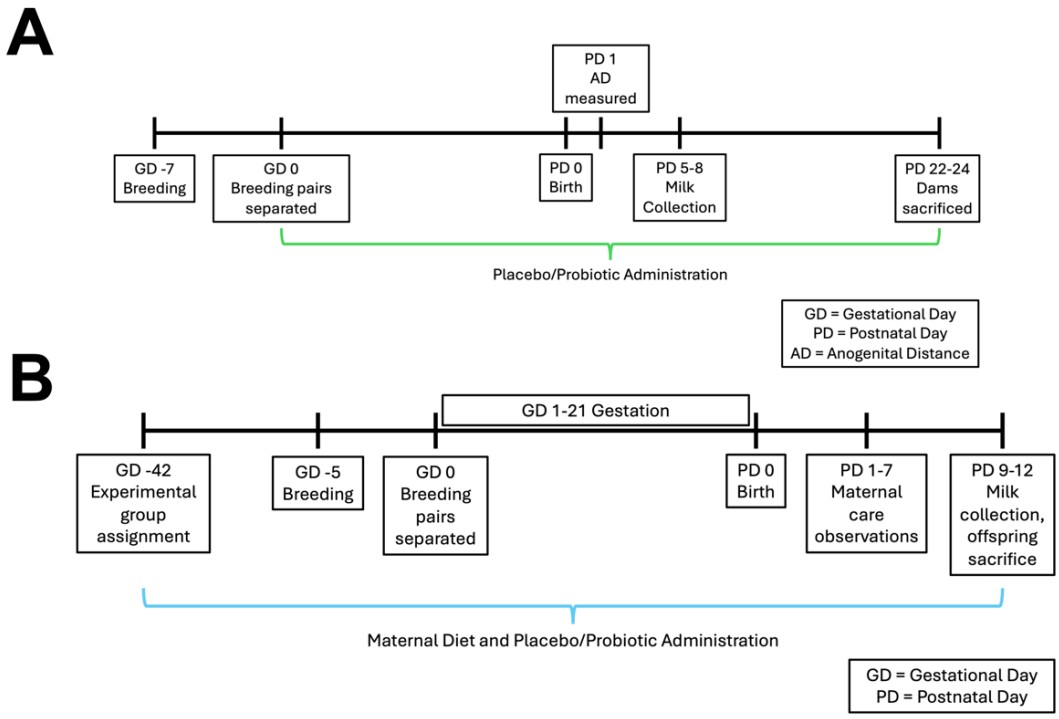

Figure 4.1 A) An experimental timeline illustrating Study 1. B) An experimental timeline illustrating Study 2.

4.1.8 Study 1: Probiotic Administration

Probiotic and placebo solutions were made fresh daily for the experiment. The probiotic was prepared at a dosage of 500 million (5×10^8) colony-forming units in reverse-osmosis water. Each rat received 0.5 mL (0.0192 grams of lyophilized powder) of solution. The administration of the placebo or probiotic occurred at 1000h $\pm$ 1 hour each day. Female breeders were trained to receive their designated placebo or probiotic solution over four days at 1000h $\pm$ 1 hour, according to a protocol outlined by Tillmann and Wegner

(2018), which had previously been used in the laboratory (Myles, O'Leary, Romkey, et al., 2020). Once a day for four consecutive days, females were trained to take 0.5 mL of their designated solution from a 1 mL oral syringe. Training was done before breeding so as not to disrupt the mating pairs. Starting on GD 0, daily probiotic (or placebo) administration began on this day. It continued through the postnatal period until the dam was sacrificed (between postpartum day [PD] 22-24 after litters were weaned).

4.1.9 Study 1: Birth Weight and Anogenital Distances

Upon reaching GD 17, dams were monitored twice daily for litters, once at 1000h and once at 1500h. Once a litter was born (PD 0), the dam and her pups were transferred to a fresh cage (with a small amount of old bedding transferred to preserve smell), and the pups were sexed and weighed. Pups' anogenital distances were measured on PD 1 using stainless steel callipers. The callipers were disinfected between the placebo and probiotic rooms with 70% ethanol, followed by Prevail® laboratory disinfectant spray (Virox® Animal Health, Virox Technologies Inc., Ontario, Canada).

4.1.10 Study 1: Milk Collection Preparation

Milk samples were collected once for each dam between PD 5-8. The dam was first separated from her offspring one hour before the milking procedure (Paul et al., 2015) and placed in a fresh cage with food and water available. The offspring were kept in the original home cage and placed on a heating pad set at low heat. Before beginning the milking procedure each day, the tabletop used for the procedure and all necessary surgical equipment was disinfected with Prevail®. A heating pad, set at medium heat, with an

absorbent bench pad overtop, was turned on before the procedure to maintain the dam's body temperature during anesthesia. All other materials needed (see Appendix H) were laid out within reach of the experimenter.

4.1.11 Study 1: Anesthetization, Oxytocin Injection and Preparation of Milking Sites

The dam was placed in an anesthesia chamber, the oxygen tank was opened, and the oxygen flow was set to 1 L (1,000 cc) per minute. The isoflurane flow was set to 5% for initial anesthesia. Once the dam was fully anesthetized (checked using pedal reflex), she was placed on her back on the heating pad. The isoflurane flow was reduced to 3-4% to maintain anesthesia, and a protective eye lubricant was applied to the corners of the dam's eyes.

Following disinfection of the vial 0.2 mL of oxytocin was injected intraperitoneally into the lower right quadrant of the abdomen, 0.5 cm deep, at an angle of 15-30 degrees. I waited approximately 10 minutes for the oxytocin to stimulate milk letdown. While waiting, the mammary glands were sterilized using a sterile alcohol pad.

4.1.12 Study 1: Milk Collection

The milking procedure commenced approximately 10 minutes after the oxytocin injection or once the milk letdown became noticeable. The mammary glands were manually squeezed upward with the thumb and forefinger until a bead of milk formed. The milk was collected from the gland with a sterile 50 μL micro-dispenser capillary tube (Kimble-Chase, Cat. #43234-2002). Once enough milk (i.e., approximately 25 μL) had

been collected, the sample was expelled into a microtube using a syringe to displace it from the capillary tube. I repeated these steps until approximately 50 – 100 μL of milk was collected. Milk samples were stored at -80ºC until further processing. After the procedure, the dam was put in a quiet room, in a fresh cage on a heating pad (set to low temperature) and kept for observation for approximately 1 hour before being returned to her pups.

4.1.13 Study 1: Sacrifice and Tissue Collection

Dams were sacrificed between P22-24. Dams were first anesthetized using Euthanyl® (sodium pentobarbital, 60 mg/kg, intraperitoneal). Once the dam was confirmed to be in surgical plane anesthesia by checking the toe-pinch reflex, the animal was quickly decapitated. All collected samples were then stored at -80ºC until further analysis.

4.1.14 Study 2: Animals and Breeding

Animal housing conditions were the same as those described above for Study 1. The information for the animals bred in this study and the resulting offspring are detailed in the methods section of Chapter 2. Briefly, dams were placed into one of four experimental groups: Placebo-Standard Diet, Placebo-Western Diet, Probiotic-Standard Diet and Probiotic-Western Diet. They were given their respective diets for 42 days before breeding and then through the breeding period (5 days), gestation (21 days) and 9-12 days postpartum. Therefore, there were approximately 11 weeks of combined WD (or standard diet, SD) and Lacidofil® probiotic (or placebo) treatment for the dams. When the offspring were born (PD 0), experimenters sexed and weighed them. The procedures performed for this study were conducted following the Canadian Council on Animal Care (CCAC)

guidelines. They were approved by the Dalhousie University Committee on Laboratory Animals (UCLA) under ethics protocol approval #20-136.

4.1.15 Study 2: Milk Collection

The milk collection procedure for Study 2 was performed according to the protocol utilized in Study 1, with a few alterations. The first alteration was that approximately 100 – 200 μL of milk was collected. The milk samples were aliquoted into two microtubes and stored at -80°C until further processing. The second alteration was that the dam was sacrificed immediately after the procedure.

4.1.16 Study 2: Sacrifice, Tissue Collection and Processing

The offspring were sacrificed between PD 9 - 12 within one hour of being separated from the dam. All rats were sacrificed with Euthanyl®, and surgical plane anesthetization was confirmed by checking that the toe pinch reflex was absent. The offspring were decapitated via sharp surgical scissors. Fresh, whole brains were collected from offspring and dams and flash-frozen in chilled isopentane (approximately -70°C). Spleen and caecum were also dissected from the dams and flash-frozen on dry ice. All tissue samples were stored at -80°C to await further processing.

Offspring brains were coronally dissected for later RNA extraction. Before each dissection, all dissection materials were cleaned first with 70% ethanol and then with RNase AWAY™ (Cat. #7000TS1, ThermoFisher Scientific). The brains were dissected by hand, using a Plexiglass® brain matrix with 1 mm slots. The brain and brain matrix were placed onto a stainless-steel dissection block and cooled using dry ice to keep the brains

frozen during this process. Using the Rat Brain Atlas as a guide, the brains were sectioned coronally using surgical stainless steel razor blades to obtain a slice of tissue with the hippocampus (Plates 30 [Bregma -2.56 mm] to 42 [Bregma -5.60 mm]; Paxinos & Watson, 1998). Once the slice was cut, it was transferred to a clean microscope slide and placed onto the dissection block. Using a scalpel and tweezers, the hippocampus was cut out of the tissue slice and placed in a clean microtube. These samples were then stored at -80°C.

4.1.17 Study 2: Plasma Collection and Preparation

At sacrifice, whole trunk blood was collected in a microtube containing 15 µL of sodium heparin (DIN 02303086, Sandoz Canada Inc, Boucherville, QC) and placed on ice immediately until plasma could be processed later that day. All plasma samples were prepared by centrifuging the whole blood at 4°C for 15 minutes at 1,000 × *g*. The supernatant was collected and pipetted into a new microtube, followed by a second centrifugation at 4°C for 10 minutes at 10,000 × *g*. The plasma from the second centrifugation was pipetted into a new microtube for long-term storage at -80°C until assayed.

4.1.18 Study 2: RNA Protocols

4.2.12.1 Offspring Hippocampal RNA Extraction

RNA was extracted from gross coronal dissections of offspring hippocampi using the RNeasy® Lipid Tissue Mini Kit (Cat. #74804, QIAGEN). First, the samples were homogenized in autoclaved bead-beating tubes that contained four 3 mm glass beads (Cat. #11.312A, Fisher Scientific®) and 1 mL of QIAzol Lysis Reagent. Using a vortex adaptor

(Cat. #SIH524, Scientific Industries, Inc.), the samples were vortexed at full speed for 1 minute, with 30 seconds of rest, for five cycles. All samples were visually inspected to ensure the tissue was fully homogenized (i.e., no longer visible). I proceeded to follow the manufacturer's instructions for the RNeasy® Lipid Tissue Mini Kit, with the following exceptions: all centrifuge steps were run for 1 minute at 12,000 × *g,* and I performed the optional membrane-drying step before eluting the samples in RNase-free water.

4.2.12.2 Offspring Hippocampal RNA Purity and Quality Measurements

RNA was quantified via spectrophotometry (Cat. #ND8000LAPTOP, Nanodrop™ 8000 Spectrophotometer, ThermoFisher Scientific) to determine its purity using the 260/280 nm ratio. The RNA quality was ascertained by checking a few samples randomly, using the Agilent Bioanalyzer RNA 6000 Nano System chip, as per the manufacturer's instructions (Cat. #5067-1511, RNA 6000 Nano Kit, Agilent Technologies).

4.2.12.3 Offspring Hippocampal DNase Treatment for RNA

First, the volume of RNA (µL) and RNase-free water (µL) required for each sample to be normalized to the final RNA concentration to 4 µg was calculated. Next, the ep*Motion® 5075t* liquid handling robot (Cat. #5075006022, Eppendorf) was programmed with these values to normalize each sample in a new microtube. After normalization, 2 µL of 10X TURBO DNase Buffer and 3 µL TURBO DNase were manually added to each sample (TURBO DNA-free™ Kit, Cat. #AM1907, Invitrogen). The samples were vortexed and subsequently incubated at 37ºC for 25 minutes. Then, 3 µL of resuspended DNase Inactivation Reagent (TURBO DNA-free™ Kit, Cat. #AM1907, Invitrogen) was added to each tube, vortexed and incubated at 25ºC for 5 minutes, with intermittent mixing by flicking each tube. Post-incubation, the samples were centrifuged at 12,000 × *g* for 2

minutes at RT. Lastly, 21 µL of the supernatant was transferred into a new microtube, and samples were stored at -20ºC.

4.2.12.4 Offspring Hippocampal qPCR of DNase-Treated RNA

A subset of the DNase-treated RNA samples (i.e., 16 of 65 samples) were checked for gDNA contamination using quantitative PCR (qPCR) with the RPL13A primer (Langnaese et al., 2008). The master mix was made by multiplying the following amounts by the number of wells needed + 15% to account for pipetting error and dead volume: 12.5 µL of SYBR Select Mix (2X), 0.075 µL of 100 µM forward primer stock, 0.075 µL of 100 µM reverse primer stock and 11.35 µL of PCR-grade, RNAse-free water.

Then, 24 µL of master mix was loaded with 1 µL of sample (per well) into a 96-well plate (Cat. #HSP9601, Bio-Rad). The samples and negative controls (master mix with RNase-free water) were assayed in singlicate using the CFX96 Touch Real-Time PCR Detection System (Cat. #1845097, Bio-Rad) with the following cycling conditions: 1 repetition of 50°C for 2 minutes; 1 repetition of 95°C for 2 minutes; 40 repetitions each of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; followed by dissociation at 95°C for 10 seconds, 65°C for 31 seconds, ten repetitions of 65°C for 5 seconds + .5°C/cycle Ramp .5°C/second. Once the run was completed, all samples were checked for amplification (indicative of gDNA contamination) using the CFX Maestro Software (Cat. #12013758, Bio-Rad). No samples were found to be contaminated.

4.2.12.5 Offspring Hippocampal cDNA Synthesis

First, the DNase-treated RNA was normalized to 2 µg in a new microtube. Subsequently, 2 µL oligo dT (50 µM; Integrated DNA Technologies) and 2 µL 10mM dNTP mix (Cat. #10297018, Invitrogen, ThermoFisher Scientific) were added to each tube,

and the samples were incubated at 65ºC for 5 minutes and then immediately transferred onto ice for 1 minute.

After the ice incubation, 4 µL of 5X first strand buffer, 1 µL DTT (0.1 M) and 1 µL Superscript IV enzyme (Superscript™ Reverse Transcriptase, Cat. #18090010, ThermoFisher Scientific) was added to each sample. Then, samples were incubated at 50ºC for 50 minutes, following by heating samples to 85ºC for 5 minutes to inactivate this reaction. The inactivation was followed by adding 1 µL of RNase mix (1 µL RNase A Cat. #12091021, Sigma, 10 µL RNase H, Cat. #10786357001, Millipore Sigma, and 389 µL RNase-free water) to the samples, which were then incubated at 37ºC for 30 minutes. The cDNA samples were then stored at -20ºC.

4.2.12.6 Offspring Hippocampal Reverse-Transcription Quantitative PCR (RT-qPCR)

The cDNA was diluted to 1:5 to assay the samples using the Ep*Motion® 5075t* liquid handling robot. All reactions, regardless of the primer, were performed in a 10 µL reaction volume (9 µL Master Mix, 1 µL cDNA or 1 µL control) in a 384-well plate (Cat. # HSP3805, Bio-Rad) and were loaded into the plate by the liquid handling robot. The samples and the controls were run in triplicate for each primer (rGR as the gene of interest, GAPDH and RPL13A as reference genes; see Appendix I for primer sequences, product sizes and melt temperature). The samples were run on two 384-well plates, so each sample's gene of interest and both reference genes were run on the same plate. Each 384-well plate was counterbalanced for sex, maternal treatment, and maternal diet. The primers used are published and have been validated by my laboratory previously: rGR (Mashoodh et al., 2009), GAPDH (Z. Li et al., 2009) and RPL13A (Langnaese et al., 2008; Myles, 2019). Plates were run in the CFX384 Touch Real-Time PCR System (Bio-Rad Laboratories,

Hercules, USA). The PCR efficiency, amplification curves and quantification cycle (Cq) values were obtained using the CFX Maestro Software (Cat. #12013758, Bio-Rad) and exported to Microsoft Excel (Microsoft Excel for Mac, Version 16.84) for further analysis.

4.2.12.7 Immunoassay Protocols

Plasma Corticosterone Enzyme Immunoassay (ELISA). The plasma samples for this assay were prepared identically to the assay performed in Chapter 2, using the same type of assay kit (DetectX® Corticosterone Enzyme Immunoassay Kit (Cat. #K014-H1, Arbor Assays, MI, USA). The protocol for this assay is detailed in Chapter 2. The same methods were used for this experiment.

Leptin Enzyme-Linked Immunosorbent Assay (ELISA). Plasma samples used in this assay were first thawed on ice and then diluted to a 1:5 ratio using the standard diluent buffer from the Rat Leptin ELISA Kit (Cat. #KRC2281, Invitrogen). Milk samples were thawed on ice and thoroughly vortexed before diluting to a 1:10 ratio using the standard diluent buffer from the Rat Leptin ELISA Kit. The ELISA assay was performed according to the manufacturer's instructions, and the plate was read using a microplate reader (Multiskan FC, ThermoFisher Scientific) at 450 nm using SkanIt™ software (Research Edition, Version 6.0.2, ThermoFisher Scientific).

4.1.19 Statistical Analyses

For both studies, statistical analyses were performed using Jamovi (Version 2.3.21.0; Fox & Weisberg, 2020; Love et al., 2022; R Core Team, 2021). Any values obtained were considered outliers if they were three times the standard deviation above or below the mean.

4.2.13.1 Study 1 Statistical Analyses

Student's t-tests were performed to assess the leptin and insulin concentrations in dams' breast milk in placebo-treated (n = 5) versus probiotic-treated dams (n = 5), with significance set at $p < .05$. Offspring birth weights and anogenital distances were analyzed using two-way factorial ANOVAs, with maternal treatment (placebo [n = 106] versus probiotic [n = 86]) and offspring sex (male [n = 106] versus female [n = 86]) as factors, significance set at $p < .05$.

4.2.13.2 Study 2 Statistical Analyses

All offspring measures, except for the RT-qPCR data for GR expression, were analyzed using factorial ANOVAs, with maternal diet (SD versus WD), maternal treatment (placebo versus probiotic) and sex (female versus male) as the factors, with significance set at $p < .05$. For all factorial ANOVAs, Tukey's *post hoc* comparisons were performed when needed. The results from the corticosterone and leptin ELISAs were exported to Microsoft Excel (Microsoft Excel for Mac, Version 16.84) for further analysis. It was decided *a priori* that samples with CVs greater than 20% would be excluded from statistical analysis. Outliers were removed if they exceeded 3 standard deviations above the mean. Several samples obtained a signal for the leptin ELISA but were below the detection range. For these samples, their values were extrapolated by solving for "x" in the following equation (utilizing an online equation solver from www.wolframalpha.com), obtained from the plate reader software:$y = a + (\frac{b-a}{1} + \left(1 + \frac{x}{c}\right)^{d})$. The sample sizes for each offspring measure are summarized in Table 4.1.

The RT-qPCR results for statistical analyses were calculated using the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001) when comparing offspring in the following

experimental groups: Maternal Probiotic Treatment versus Placebo, Western Diet versus Standard Diet and Male versus Female. One-way ANOVAs compared the transformed ΔCq values for Treatment, Diet and Sex, with significance set at $p < .05$.

Milk leptin concentrations were analyzed using factorial ANOVA, with maternal treatment (placebo versus probiotic) and maternal diet (SD versus WD) as factors (see Table 4.2 for sample sizes). The significance level was set at $p < .05$.

Table 4.1 Sample sizes for Study 2 offspring measures, including offspring birth weight, GR expression, plasma corticosterone concentration and plasma leptin concentration.

Sex	Maternal Treatment	Maternal Diet	Offspring Birth Weight *n*	GR Expression *n*	Plasma Corticosterone *n*[a]	Plasma Leptin *n*[a]
Male	Placebo	SD	47	7	7	7
		WD	36	9	4	9
	Probiotic	SD	52	7	7	9
		WD	34	9	7	8
Female	Placebo	SD	40	6	8	7
		WD	37	9	8	9
	Probiotic	SD	53	7	8	9
		WD	37	8	5	9

[a]The same probiotic WD male was removed as an outlier in both measures.

Table 4.2 Sample sizes for milk leptin concentration in Study 2.

Treatment	Diet	*n*
Placebo	Standard	6
	Western	6
Probiotic	Standard	8
	Western	8

RESULTS

4.1.20 Study 1: Offspring Birth Weight and Litter Sizes

Two main effects on offspring birth weight were revealed: maternal treatment and offspring sex. For maternal treatment, offspring from probiotic-treated dams weighed more at birth (M = 7.02; SD = 0.72; $F_{1,188}$ = 5.28, $p < .023$, η^2 = 0.026) compared to offspring from placebo-treated dams (M = 6.82; SD = 0.58; see Figure 4.2A). For sex, male offspring (M = 7.02; SD = 0.61; $F_{1,188}$ = 6.87, $p < .010$, η^2 = 0.034) weighed more than female offspring (M = 6.77; SD = 0.69). No differences in litter size were observed based on maternal treatment.

4.1.21 Study 1: Offspring Anogenital Distance

Two main effects on offspring anogenital distance were revealed: maternal treatment and offspring sex. First, male offspring had greater anogenital distances (M = 3.00; SD = 0.38; $F_{1,188}$ = 855.87, $p < .001$, η^2 = 0.812) compared to females (M = 1.44; SD = 0.37). Offspring from probiotic-treated dams also had greater anogenital distances (M =

2.38; *SD* = 0.88; $F_{1,188}$ = 9.29, *p* = .003, η^2 = 0.009) compared to offspring from placebo-treated dams (*M* = 2.23; *SD* = 0.85; Figure 4.2B).

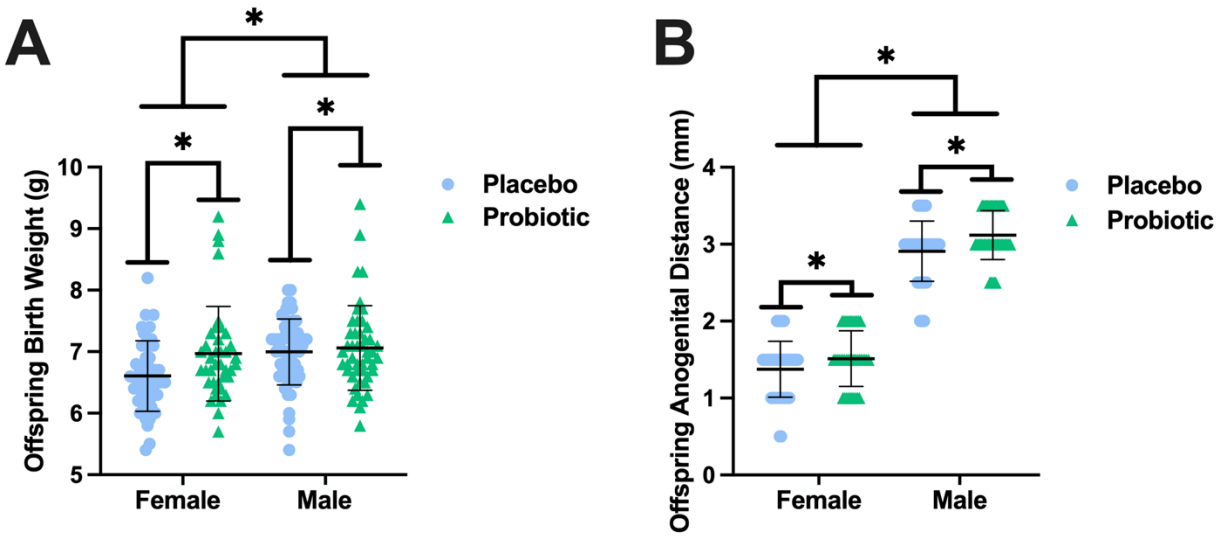

Figure 4.2 A) Offspring birth weights, in grams, separated by sex (female versus male) and maternal treatment (placebo versus probiotic). B) Offspring anogenital distances, in millimetres (mm), separated by sex (female versus male) and maternal treatment (placebo versus probiotic).

4.1.22 Study 1: Leptin and Insulin ELISAs on Breast Milk

No significant group differences were revealed for leptin or insulin levels in the breast milk samples from probiotic-treated (*M* = 832.00, *SD* = 762.00) versus placebo-treated rats (*M* = 1310.00, *SD* = 928.00).

4.1.23 Study 2: Offspring Birth Weight and Litter Sizes

There were two main effects on offspring birth weights. The first is that offspring sex impacted birth weight ($F_{1,328}$ = 62.71, *p* < .001, η^2 = 0.149), such that male offspring were heavier at birth (*M* = 6.63; *SD* = 0.55) compared to female offspring (*M* = 6.17, *SD* =

0.51; see Figure 4.3A). The second main effect was of maternal diet ($F_{1,328} = 25.51$, $p < .001$, $\eta^2 = 0.061$), where offspring born to dams fed a WD weighed less at birth ($M = 6.23$; $SD = 0.56$), compared to their counterparts from SD-fed dams ($M = 6.52$; $SD = 0.56$; see Figure 4.3B).

I also examined whether maternal diet and probiotic treatment affected litter sizes and sex ratios. Maternal WD litters were significantly smaller ($M = 9.60$; $SD = 3.16$; $F_{1,27} = 42.59$, $p = .035$, $\eta^2 = 0.140$) compared to litter sizes from dams provided SD ($M = 12.0$; $SD = 2.85$; see Figure 4.3C). There was no significant difference in the number of female or male offspring by maternal diet, treatment, or interaction.

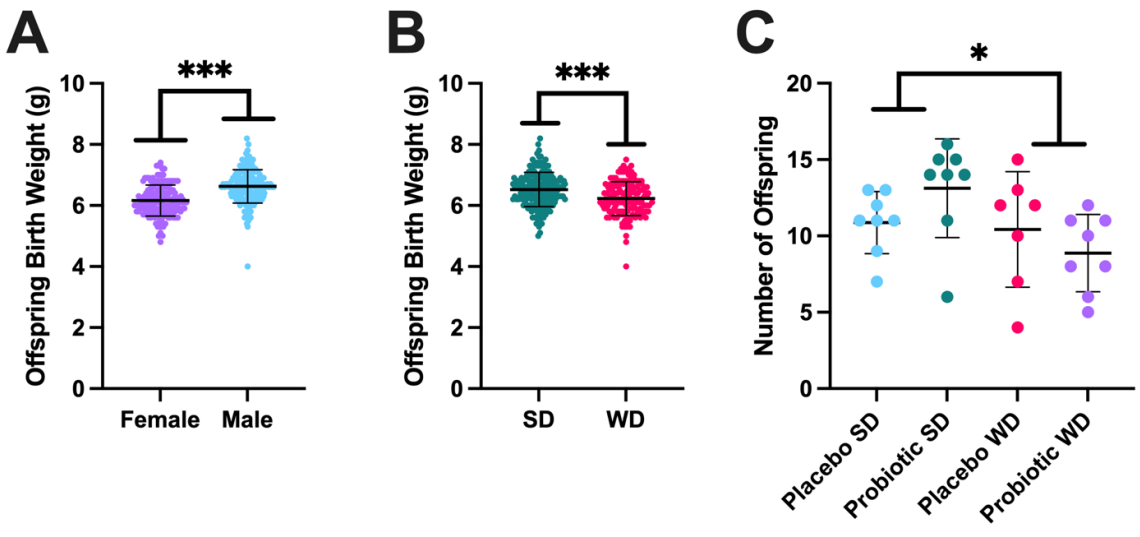

Figure 4.3 A) Main effect of sex on offspring birth weights (in grams), $p < .001$; B) Main effect of maternal diet on offspring birth weights (in grams), $p < .001$. C) Main effect of maternal diet on litter size (the number of offspring born on PD 0), $p < .05$.

4.1.24 Study 2: Glucocorticoid Receptor Expression in Offspring Hippocampi

First, using an average of the quantification cycles (Cq) for each sample, I calculated the expression fold change (ΔCq) for each independent variable of the offspring: Treatment (Placebo versus Probiotic), Diet (Standard versus Western) and Sex (Male versus Female). The fold change was calculated using the averaged Cq values from the two reference genes (RPL13A and GAPDH). As previously stated, to obtain the transformed ΔCq values to perform statistical analyses, I utilized the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001). There were no observed differences in hippocampal glucocorticoid receptor expression based on offspring sex, maternal treatment or maternal diet. The transformed ΔCq values for glucocorticoid receptors for each independent variable are shown in Figure 4.4.

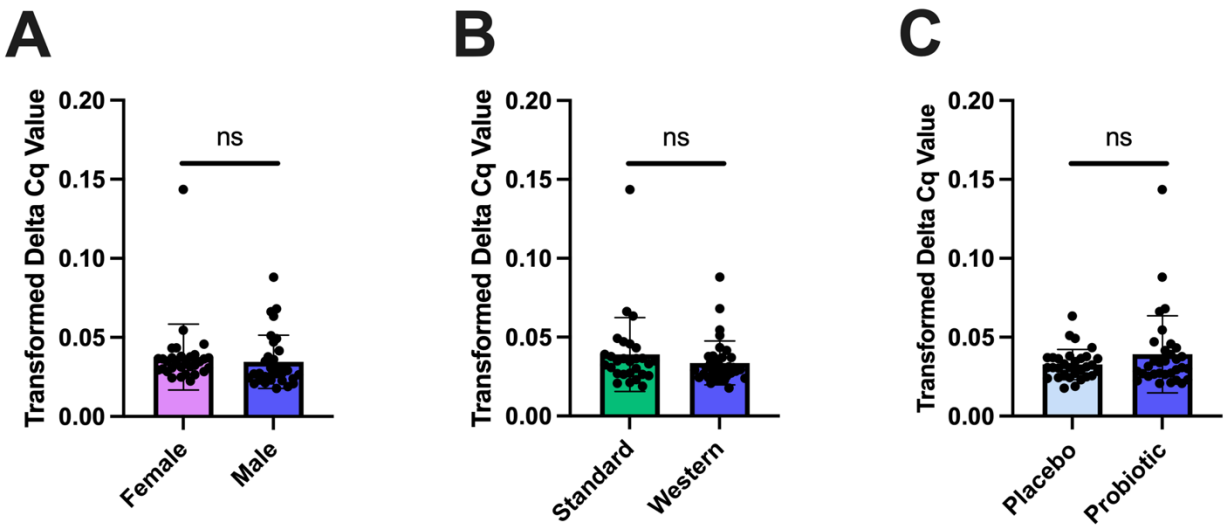

Figure 4.4 A) The transformed relative normalized expression of glucocorticoid receptors from mRNA extracted from gross coronal dissections of offspring hippocampus for A) offspring sex (female vs. male), B) diet condition (Standard vs. Western diet) of offspring's dams and C) treatment condition (placebo vs. probiotic) of offspring's dams.

4.1.25 Study 2: Offspring Plasma Corticosterone Concentrations

A 2 × 2 × 2 factorial ANOVA assessing maternal treatment, maternal diet, and offspring sex revealed a significant main effect of diet ($F_{1, 46} = 8.04$, $p = .007$, $\eta^2 = 0.111$) and a significant interaction between treatment and diet ($F_{1, 46} = 8.30$, $p = .006$, $\eta^2 = 0.115$; see Figure 4.5A) on offspring plasma corticosterone levels.

Using Tukey's *post hoc* comparisons to probe the treatment by diet interaction, it was revealed that the offspring from probiotic-treated WD dams had significantly higher plasma corticosterone levels ($p_{Tukey} = .015$; $M = 19{,}279.00$; $SD = 16{,}577.00$) compared to offspring from placebo-treated, SD dams ($M = 10{,}133.00$; $SD = 3{,}781.00$) and offspring from placebo-treated, WD dams ($p_{Tukey} = .026$; $M = 10{,}588.00$; $SD = 5{,}492.00$). The offspring from the probiotic-treated WD dams also had significantly higher plasma corticosterone levels than their counterparts from probiotic-treated SD dams ($p_{Tukey} < .001$; $M = 7{,}026.00$; $SD = 2{,}299.00$).

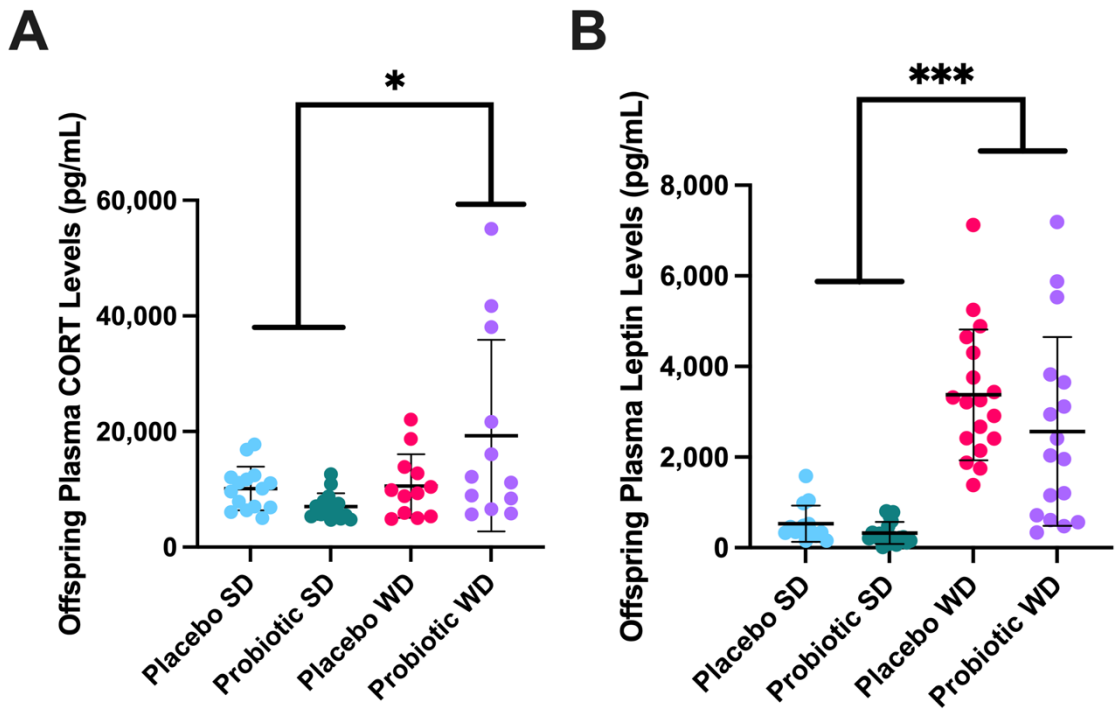

Figure 4.5 A) Offspring plasma corticosterone (CORT; mean, SD) concentrations in pg/mL, $p < .05$. B) Offspring plasma leptin concentrations (pg/mL) show diet's main effect on leptin levels, $p < .001$.

4.1.26 Study 2: Offspring Plasma Leptin Concentrations

When I measured the plasma leptin levels in the offspring, I found a main effect of diet ($F_{(1,69)}$ =56.39, $p < .001$, $\eta^2 = .433$) where offspring from dams fed a WD had significantly higher plasma leptin (M = 3,258.00; SD = 2433.00) compared to their counterparts from SD-fed dams (M = 328.00; SD = 326.00; see Figure 4.5B). I did not observe any main effects of treatment or sex, nor were there any interactions.

4.1.27 Study 2: Dam Breast Milk Leptin Concentrations

For leptin concentrations in dams' breast milk, I found a main effect of treatment ($F_{1,24}$ = 8.46, p = .008, η^2 = 0.209) where dams provided probiotic treatment had significantly lower leptin levels in their milk (M = 643.00; SD = 193.00) compared to their placebo-treated counterparts (M = 844.00, SD = 216.00; see Figure 4.6A). I also observed a main effect of diet on milk leptin levels ($F_{1,24}$ = 7.50, p = .011, η^2 = 0.186), such that WD-fed dams had significantly more leptin in their milk (M = 828.00; SD = 222.00), compared to dams fed SD (M = 632.00; SD = 183.00; see Figure 4.6B).

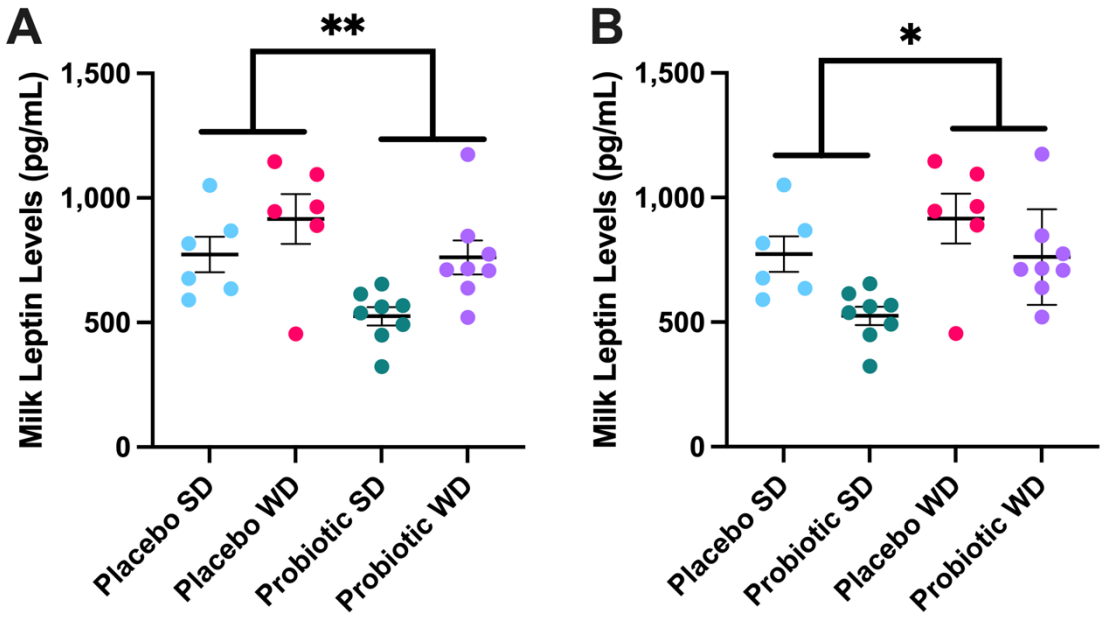

Figure 4.6 A) Dam breast milk leptin concentrations (pg/mL) show the main effect of treatment on leptin levels, $p < .01$; B) Dam breast milk leptin concentrations (pg/mL) show the main effect of diet on leptin levels $p < .05$.

DISCUSSION

The results for both Study 1 and Study 2 are summarized in Table 4.3.

Table 4.3 Summary of experimental results for Studies 1 and 2.

	Summary of Experimental Results
Study 1	• Increased offspring birthweight of probiotic-treated dams • Increased offspring anogenital distance of probiotic-treated dams
Study 2	*Offspring Outcomes* • Decreased offspring birthweight from WD-fed dams • Decreased litter size in WD-fed dams • Increased corticosterone in offspring of probiotic-treated WD-fed dams • Increased leptin in offspring from WD-fed dams *Dam Outcomes* • Increased milk leptin from WD-fed dams • Decreased milk leptin from probiotic-treated dams

Study 1 found that offspring exposed to maternal probiotic treatment weighed more at birth than offspring exposed to maternal placebo. It was found that offspring from probiotic-treated dams had increased anogenital distances on PD 1 compared to offspring from placebo-treated dams. No differences were found for leptin and insulin levels in breast milk samples from placebo- versus probiotic-treated dams.

Study 2 found that offspring born to WD-fed dams weighed significantly less at birth than offspring from SD-fed dams. Maternal WD also decreased litter size but did not impact the sex ratio. When offspring plasma corticosterone concentrations were examined, a significant interaction between maternal treatment and diet was observed, such that offspring born to probiotic-treated, WD-fed dams had increased levels of corticosterone compared to their counterparts from probiotic-treated, SD-fed dams. No differences in glucocorticoid receptor gene expression levels in the hippocampi of offspring were found.

Offspring from WD-fed dams had significantly increased plasma leptin concentrations compared to offspring from SD-fed dams. Lastly, it was found that probiotic treatment significantly lowered leptin levels in breast milk, whereas maternal WD increased leptin. There was no interaction between treatment and diet for milk leptin levels.

4.1.28 Maternal Probiotic Treatment and Diet (Separately) Affect Birth Weights

Study 1 found that maternal probiotic administration resulted in higher birth weights than offspring born to placebo-treated dams. My previous work with the probiotic Lacidofil® found that treatment did not impact birth weights but resulted in higher weaning weights than placebo-exposed offspring (O'Leary, 2019). In a study using female Sprague-Dawley rats, maternal probiotic administration of *L. rhamnosus* GR-1 and *L. fermentum* RC-14, it was found that these probiotics increased birth weight compared to controls (Anukam et al., 2005). In a study involving pregnant women, those with higher levels of endogenous salivary *L. casei* bacteria were positively associated with birth weight and gestational age (Dasanayake et al., 2005). These results are consistent with the current study's findings that the probiotic Lacidofil® increases birth weight. Furthermore, there is evidence for the use of probiotics as a treatment in cases of low-birth-weight infants. In a meta-analysis investigating the effects of probiotic therapy on mortality in low-birth-weight infants, it was found that a combination of *Lactobacillus* and *Bifidobacterium* species had high certainty for reducing mortality and stage two necrotizing enterocolitis, compared to placebo treatment (Morgan et al., 2020).

Study 2 found that offspring from WD-fed dams had low birth weights, which was inconsistent with the original hypothesis. This is consistent with a 2003-2006 pregnancy

study in the United Kingdom found that total dietary fat consumption was linked to lower birth weights (Sharma et al., 2018).

Low birth weight is a risk factor for later adulthood health issues, including chronic heart defects and chronic bronchitis (Barker 1998 as cited by Roseboom et al., 2001). As previously discussed, the concept of DOHaD demonstrates that gestation, birth, and adulthood health are linked via early-life programming (Roseboom et al., 2001; Suzuki, 2018). Through the Dutch Famine Birth Cohort Study, it was found that famine exposure during early gestation resulted in the increased likelihood of several potential health issues, including higher BMI, higher risk of chronic heart defects and an atherogenic lipid profile (Roseboom et al., 2000). It is postulated that the mismatch between undernutrition during early gestation and adequate nutrition postnatally may have resulted in these metabolic programming issues in adulthood (Roseboom et al., 2001). Although the research on whether or not maternal Western diet results in lower birth weights is mixed (Gete et al., 2020), there is evidence that maternal diet does affect fetal growth (Kjøllesdal & Holmboe-Ottesen, 2014).

A review of human maternal dietary patterns showed that diets with greater consumption of processed foods were positively associated with increased instances of low-birth-weight children (Kjøllesdal & Holmboe-Ottesen, 2014). This finding is consistent with previous research demonstrating that the micronutrient content of a mother's diet is important for fetal growth (Lagiou et al., 2005; Rao et al., 2001). Conversely, dietary patterns associated with average infant birth weights were characterized by the consumption of more fruits, vegetables and dairy products (Kjøllesdal & Holmboe-Ottesen, 2014). Another study found that mothers who consumed higher

confections and condiments (i.e., high-sugar foods) had higher instances of low-birth-weight infants (Loy et al., 2013). Similarly, other human studies have found that maternal hypertension, which is linked to Western diet consumption, was also associated with low infant birth weight (Boo, 2008; Rahman et al., 2008).

Additionally, there is evidence from animal studies demonstrating a link between maternal Western diet consumption and low birth weight. In a study of Wistar rats fed either a standard chow or Western diet (45% kcal fat, Cat. # D12451, Research Diets) before and during pregnancy, researchers found that offspring born to WD-fed dams weighed less at birth (Howie et al., 2009). In another study of Sprague-Dawley rats provided with either a standard or Western diet (45% kcal fat, Research Diets) for 16 weeks before breeding, WD-fed dams showed reduced fetal growth, and their offspring had lower birth weights compared to controls (Hayes et al., 2012). Researchers then investigated the dams' placental blood vessels (Hayes et al., 2012). They found that although WD-fed dams had more blood vessels than controls, the number of blood vessels was inversely related to the amount of smooth muscle cell actin present (i.e., an indicator of blood vessel maturity; Cîmpean et al., 2007). Therefore, researchers suggested that this reduction in mature blood vessels is involved in the increased instances of low-birth-weight offspring, as they are likely not receiving sufficient blood flow for proper development (Hayes et al., 2012). This is further evidenced by greater levels of carbonic anhydrase, an indicator of hypoxia, in the placental tissues of WD-fed dams (Hayes et al., 2012).

However, there is also evidence to the contrary. In a study by Song and colleagues (2015), female Sprague-Dawley rats were fed a high-fat diet (45% kcal fat, Cat. # D12451, Research Diets, Inc. USA) or a control diet (10% kcal fat, Cat. # D12450B). The offspring

born to high-fat diet females had higher birth weights, higher adult weights and increased serum leptin levels than those from control-fed dams (Y. Song et al., 2015). In human participants, a scoping review of Malaysian women showed an increased likelihood of high birth weight if maternal BMI was also high (Mohamed et al., 2022).

Therefore, there is evidence from both human and animal studies consistent with my results of higher maternal fat intake and lower birth weight. Although I did not find that maternal probiotic administration interacts with maternal diet to affect offspring birth weight, there is evidence that it can be beneficial in WD models where offspring are likely to be born large for their gestational age. In a maternal WD (30% fat) model using Wistar rats, it was found that the WD, in combination with a symbiotic solution (*Lactobacillus rhamnosus*, *Bacillus coagulant* and fructooligosaccharide), resulted in lowered offspring birth weights, compared to offspring from dams fed WD exclusively (Amirpour et al., 2020).

The summation of previous work and my current findings indicates the nuances of early-life programming and supports the idea that a U-shaped curve represents the programming of infant or offspring health outcomes, where the lightest and heaviest infants (as measured at birth) suffered the greatest risk for metabolic disease (McCance et al., 1994).

4.1.29 Maternal Probiotic Treatment Increases Anogenital Distance as Measured at Birth

To my knowledge, there are no reports of how maternal probiotic treatment may affect offspring anogenital distance at birth. Based on a previous study by Cowan and Richardson (2019) that found that probiotic treatment Lacidofil® was able to correct early-

onset puberty due to maternal stress, I wanted to explore how probiotics may impact early-life sexual differentiation.

I found that probiotic-exposed offspring had greater anogenital distances at birth than their placebo-exposed counterparts. Measuring anogenital distance is one way to investigate fetal exposure to sex hormones, such as testosterone (Drickamer, 1996). In female rodent offspring, intrauterine positioning determines anogenital distance (Vom Saal et al., 1990). When a female fetus is between two male offspring, the anogenital distance at birth is larger than if the fetus is between two other females (Vom Saal et al., 1990). The masculinization of the reproductive tract in male rodent offspring is also a function of androgen exposure during a critical window of development *in utero* (Welsh et al., 2008).

Interestingly, increasing evidence shows that microbes, including probiotics, may interact with sex hormones. As previously mentioned, microbes, including *Lactobacillus* strains, secrete β-glucuronidase, which aids in deconjugating estrogen, rendering it active (Plottel & Blaser, 2011). There is also evidence that the gut microbiota interacts with sex hormones (Kaliannan et al., 2018; Qi et al., 2021). For example, microbes have been found to affect 17β-estradiol, which, when provided to male C57BL/6 mice and ovariectomized female C57BL/6 mice, was preventative in situations of metabolic endotoxemia and low-grade inflammation (Kaliannan et al., 2018).

Probiotics are also being looked to as a treatment option for people suffering from polycystic ovarian syndrome (PCOS), a reproductive and metabolic disorder. In people with PCOS, insulin action increases, increasing androgen production (Teede et al., 2010). In a dihydrotestosterone (DHT)-induced PCOS model in female Wistar rats, it was shown that the administration of a multi-strain probiotic (*Bifidobacterium, Lactobacillus*

acidophilus and *Enterococcus faecali*) improved outcomes, including lowered serum cholesterol and increased serum progesterone (F. Zhang et al., 2019). Another rodent PCOS model found that *Lactobacillus* (strain not specified) treatment lowered gut levels of *Prevotellaceae* bacteria and decreased androgen levels (Guo et al., 2016). These results suggest that *Lactobacillus* bacteria interact with the endocrine system, providing stabilizing benefits.

Regarding the current results, it is unclear why probiotic treatment with Lacidofil® would increase anogenital distances in offspring, as it implies that prenatal circulating androgen levels would have been elevated in probiotic-treated dams throughout their pregnancies. In a non-obese diabetic (NOD) mouse model, researchers found that germ-free female NOD mice had increased testosterone levels compared to specific pathogen-free female mice (Markle et al., 2013). Interestingly, the germ-free male NOD mice had lower testosterone levels than the specific pathogen-free males (Markle et al., 2013). These results indicate that microbes are at least partly responsible for sex hormone expression and that microbes impact sex hormones in a sex-dependent way. Therefore, I may be seeing the effects of their exposure to the probiotic bacteria in the probiotic-exposed offspring. However, these effects are not fully realized, as the offspring have yet to experience puberty. The expression of secondary sexual characteristics at puberty and testosterone measurements would provide a more comprehensive picture of how the probiotic Lacidofil® may influence sex hormones in the offspring.

4.1.30 Offspring Plasma Corticosterone, Glucocorticoid Receptors and Leptin Concentrations

Previously, I found that using Lacidofil® as a probiotic treatment for rat dams throughout pregnancy and lactation resulted in higher frequencies of active maternal care behaviours, including arched-back nursing and licking/grooming (O'Leary, 2019). Previous research has shown that increased active maternal care is beneficial for developing the offspring's stress response, increasing GR levels in the hippocampus (Weaver et al., 2004).

The present study explored the impact of maternal probiotic treatment (with Lacidofil®) in rat dams fed a WD and the subsequent outcomes of their offspring's stress and metabolic programming. Based on previous research showing that a WD can be detrimental to maternal care behaviour and, therefore, potentially harmful to offspring, it was hypothesized that offspring from WD-fed, probiotic-treated dams would have increased hippocampal GR expression compared to offspring from WD-fed, placebo-treated dams. I also hypothesized that the offspring from SD-fed dams would have the highest hippocampal GR expression relative to the other experimental groups. I found no differences in the expression of hippocampal GR based on maternal diet, maternal probiotic treatment, or offspring sex.

Interestingly, although I found no differences in GR expression, I did observe a difference in offspring corticosterone levels. I found that the offspring born to WD-fed, probiotic-treated dams had increased levels of corticosterone compared to their counterparts from SD-fed, probiotic-treated dams.

Little research exists on how maternal diet and probiotic treatment might influence GR expression. Like my results, a study using male Wistar rats fed either a standard chow, a high-fat diet (total fat content not specified), or a high-sucrose diet for four weeks found no diet-induced differences in GR gene expression in the hippocampus (Gergerlioglu et al., 2016). Unlike my results, however, the researchers found no diet-induced differences in plasma corticosterone levels (Gergerlioglu et al., 2016).

Due to the demonstrated effects of maternal high-fat diet increasing maternal plasma corticosterone levels, as well as potentially increasing fetal corticosterone exposure due to the disruption of enzyme activity (Bellisario et al., 2015), I had hypothesized that the offspring exposed to WD via their dams would have increased plasma corticosterone. I also hypothesized that maternal probiotic treatment may mitigate the effects of the WD on elevating corticosterone levels due to previous research findings. An acute probiotic treatment (*L. helveticus* R0052 and *B. longum* R0175) in chronically stressed male mice normalized their corticosterone levels post-stressor, compared to animals that only received the vehicle, which had significantly increased corticosterone plasma levels afterwards (Ait-Belgnaoui et al., 2018). The probiotic treatment prevented a decrease in GR gene expression in the hypothalamus and hippocampus observed in the control group (Ait-Belgnaoui et al., 2018). It is worth noting that these probiotic effects may be due to the direct administration to these animals. Conversely, in a 129/SvEv mouse model, wild-type mice fed a WD (33% fat, 49% carbohydrates, 28% protein) and *Lactobacillus helveticus* R0052 had increased corticosterone concentrations in their forebrains, compared to wild-type mice provided the probiotic and regular chow (Ohland et al., 2013). These results from Ohland and colleagues (2013) are consistent with my finding that the offspring

from WD-fed, probiotic-treated dams had increased plasma corticosterone. However, it must be noted that in the current study, only the rat dams received probiotic treatment, meaning that the offspring were indirectly exposed. Additionally, although WD can be considered a physiological stressor (Bellisario et al., 2015), probiotic treatment may be more effective under specific stress conditions and not others. The differences in the programming of the stress response concerning the interplay between the gut-brain-microbiota axis may be best observed in more extreme scenarios, as well. For example, in germ-free (GF) versus specific pathogen-free (SPF) male BALB/c mice, it was found that the GF mice had increased GR pathway gene expression in their hippocampi, compared to their SPF counterparts, indicating that the lack of microbes plays a role in gene regulation (Luo et al., 2018). Furthermore, GF mice had higher plasma ACTH and corticosterone levels post-acute stress than SPF mice (Luo et al., 2018). However, it is essential to note that there were no differences in basal levels of corticosterone between the two groups (Luo et al., 2018). In an experiment using male and female adolescent CD-1 mice, it was found that probiotic supplementation (*L. lactis*, *L. cremoris*, *L. diacetylactis and L. acidophilus*) aided in mitigating LPS-induced changes in GR gene expression in the hypothalamus (K. B. Smith et al., 2021). Specifically, LPS exposure decreased GR expression in the paraventricular nucleus of the hypothalamus in male mice, whereas probiotic-treated males did not have this LPS-induced decrease in GR expression (K. B. Smith et al., 2021).

In summary, evidence suggests that gut microbes influence stress response programming. However, it may only be observable when comparing two disparate groups who have had their gut microbiota manipulated directly. In the present study, the differences in the offspring microbiota (and therefore possibly GR expression) may not

have been fully formed due to the age of the offspring (approximately ten days old) at the time of sacrifice and because they did not directly receive either the diet or the probiotic.

4.1.31 Probiotics, Diet and Breast Milk – Maternal Programming of Offspring Leptin

Study 2 found that the offspring of dams fed a WD had higher plasma leptin concentrations than their counterparts from SD-fed dams. Related to this finding, I also observed that WD-fed dams had significantly higher breast milk leptin concentrations than SD-fed dams. Although leptin levels in breast milk are typically lower than concentrations in plasma (Garcia-Mayor et al., 1997; Houseknecht et al., 1997), these results are consistent with previous research investigating breast milk leptin concentrations and offspring plasma leptin levels. For example, a study on infants and maternal breast milk composition found that the infant's plasma leptin levels were significantly correlated with the breast milk leptin levels that the infants had consumed on the same day (Chatmethakul et al., 2022). In young rats, it has also been found that leptin can be ingested orally and subsequently circulated throughout the body (Casabiell et al., 1997; Sánchez et al., 2005).

Relatedly, research shows that the infant's food *source* also impacts their leptin levels. In a study of healthy infants who were either exclusively breastfed or exclusively formula-fed, the formula-fed infants had a significantly higher amount of plasma leptin at six months old than their breastfed counterparts (Lönnerdal & Havel, 2000). Interestingly, the insulin-glucose ratios of the infants were higher in the formula-fed cohort than in the breastfed cohort at four months of age (Lönnerdal & Havel, 2000). Unlike the present work, researchers also found a sex difference in leptin levels, where female infants had significantly higher leptin levels than males (Lönnerdal & Havel, 2000).

Due to its association with metabolic programming, leptin is an excellent marker to explore in breast milk samples (Schuster et al., 2011). Previous studies have demonstrated an association between breast milk leptin levels and weight gain during the neonatal period (Doneray et al., 2009; Miralles et al., 2006). Leptin levels also correlated with maternal BMI, which may mean infants from significantly overweight mothers may be exposed to greater leptin concentrations throughout nursing (Schuster et al., 2011). Leptin exposure throughout lactation via breast milk could have important implications for regulating body weight and food intake in adulthood. Conversely, a study of healthy, exclusively breastfed infants found that the breast milk leptin levels in mothers of obese infants were not significantly different than the milk leptin levels of mothers who had non-obese infants (Uysal et al., 2002). This result indicates maternal milk leptin may not impact infant adiposity (Uysal et al., 2002).

However, it should be noted that increased leptin levels are associated with metabolic dysfunction and a higher risk of GDM (Heerwagen et al., 2010; Vähämiko et al., 2010). Since there is the possibility that maternal leptin levels may program offspring metabolic health, it could be important to monitor maternal leptin levels in future research, including the levels found in breast milk, to understand better how leptin may influence offspring health and development (Vähämiko et al., 2013). A human study by Vahamiko and colleagues (2013) found that maternal serum leptin levels significantly increased throughout the pregnancy. The researchers found no diet or probiotic administration effect on maternal serum leptin levels (Vähämiko et al., 2013). These findings are contrary to my animal model, as I found that probiotic administration decreased leptin levels in maternal milk. In contrast, the WD increased maternal milk leptin.

4.1.32 Limitations and Future Directions

Both studies included in this chapter have limitations. In Study 1, the offspring were not examined for puberty onset once they reached adolescence but were used for a different study. This would have been insightful information regarding how maternal probiotic administration may or may not affect later sexual differentiation in offspring (Cowan & Richardson, 2019; Markle et al., 2013). Future work with probiotics in early-life development models could investigate how maternal probiotic administration affects sex hormones in infancy and later in adolescence.

In Study 2, only basal corticosterone levels were characterized when investigating offspring plasma corticosterone concentrations. Utilizing a stress paradigm (e.g., predator odour exposure; Korgan et al., 2016) may have provided better insight into whether maternal probiotic administration affects HPA axis function when offspring are exposed to a stressor rather than only looking at basal levels. If a stress paradigm was used, blood samples could have been taken before and after the stressor to examine the trajectory of corticosterone release (Niu et al., 2019).

There were limited milk samples in both Study 1 and 2. However, Study 1 was conducted as a pilot study to ensure that leptin and insulin were measurable in breast milk samples. Although detectable in breast milk, the insulin assay optimized in Study 1 was unreliable and, therefore, not investigated in Study 2. In Study 2, it is possible that there were not a sufficient number of milk samples to detect any interactions occurring between maternal diet and probiotic administration to affect leptin levels.

Future work in early-life development models that investigate probiotics as a potential treatment to combat early-life stress should aim to characterize the effects of

maternal probiotic administration across various stressors. From the results of this chapter, it is clear that probiotics and various stressors interact quite differently. Furthermore, this research also highlights the importance of investigating the effects of probiotics on breast milk since it was demonstrated that the probiotic Lacidofil® impacts leptin levels in milk, which has implications for the metabolic programming of offspring.

CHAPTER 5 DISCUSSION

SUMMARY OF FINDINGS

Most research on maternal diet and other maternal factors solely focuses on offspring-related health outcomes. While offspring outcomes are important when investigating maternal factors, the primary goal of this dissertation was to explore how diet and probiotic treatment affect the mother. Maternal health is crucial for both offspring development and maternal well-being during the perinatal period and beyond.

5.1.1 Chapter 2: Maternal Outcomes

In this study, I found that maternal diet and probiotic treatment had separate effects on maternal care behaviour. The WD-fed dams engaged in significantly more LG behaviour than SD-fed dams, and probiotic-treated dams performed significantly more ABN 2 than placebo-treated dams. Diet administration also affected the metabolic, inflammatory, and stress markers measured in this study. WD-fed dams had increased inflammatory markers, leptin, and corticosterone concentrations compared to SD-fed dams.

5.1.2 Chapter 3: Maternal Microbiome

The first study in Chapter 3 did not detect many significant changes to the maternal gut microbiome based on the administration of the probiotic Lacidofil®. However, changes in beta diversity and some differentially abundant features were detected. The majority of these differentially abundant features were detected in the placebo-treated group.

The second study of Chapter 3 incorporated maternal diet and probiotic administration together. In this study, many significant findings were due to maternal diet, including differences in alpha diversity, beta diversity, relative abundance, and differentially abundant features. A significant interaction between maternal diet and probiotic treatment was found for one of the beta diversity metrics, Unweighted UniFrac. It was found that the placebo-treated, SD-fed dams had increased Firmicutes and lower Bacteroidetes compared to the other experimental groups. Of the 13 differentially abundant features found in common between MaAslin2 and ALDEx2 analyses, MaAslin2 detected main effects of treatment and diet and two diet-by-treatment interactions. In comparison, ALDEx2 detected only main effects of diet.

5.1.3 Chapter 4: Offspring Outcomes

The first study of this chapter found that offspring born to probiotic-treated dams were significantly heavier at birth compared to offspring from placebo-treated dams. The offspring from probiotic-treated dams also had significantly greater anogenital distances on postpartum day one than their placebo counterparts.

The second study in this chapter found that offspring from WD-fed dams were significantly lighter at birth than those from standard diet-fed dams. I also found that WD-fed dams had significantly smaller litters. I observed a significant interaction between maternal treatment and diet, such that offspring born to probiotic-treated WD-fed dams had increased basal plasma corticosterone levels compared to all other experimental groups. Despite this finding, there were no differences in glucocorticoid receptor mRNA levels in offspring hippocampi. WD-fed dams' offspring had significantly higher plasma leptin

concentrations than those of SD-fed dams' offspring. This effect of diet on leptin levels was mirrored in breast milk, such that maternal WD increased milk leptin, too. Interestingly, probiotic treatment significantly decreased milk leptin levels without interacting with the maternal diet.

5.1.4 Maternal Care and Offspring Stress Response Programming

The maternal care findings are partially consistent with my previous work, showing that Lacidofil® administration can increase the frequency of arched-back nursing behaviour (O'Leary, 2019). My present findings also show that Western diet administration causes a physiological stress response in the rat dams, as evidenced by their increased basal plasma corticosterone and inflammation markers (e.g., cytokines). Despite finding increased ABN 2 behaviour in the probiotic-treated dams, this did not result in any observed differences in offspring hippocampal glucocorticoid receptor mRNA levels, as was hypothesized. It is possible that because probiotics did not increase the LG behaviour specifically, the increase in ABN 2 behaviour alone was not sufficient to change the glucocorticoid receptor levels in the offspring. Future studies could first characterize rat dams into low versus high LG-ABN categories, as previous studies have (Caldji et al., 1998; Francis et al., 1999; Weaver et al., 2004). Subsequently, the rat dams could be given probiotics before breeding a second time. This baseline characterization of a rat dam's maternal care behaviour would provide new insight into how later probiotic administration affects this behaviour.

To my knowledge, this and my previous work (O'Leary, 2019) are the first studies investigating how maternal probiotic treatment affects maternal care behaviours across the first postpartum week. A previous study of maternal probiotic administration only utilized a pup retrieval test to measure maternal care behaviour (Cowan et al., 2016) rather than conducting a daily behavioural observation protocol during the early postpartum period (Francis et al., 1999; Francis et al., 2003; Weaver et al., 2004). A similar early postpartum observation protocol has been previously utilized in the laboratory (Korgan et al., 2016, 2018).

Previous research has shown that Lacidofil® can be provided to dams to mitigate the effects of early-life stress by preventing stress-induced emotional dysregulation (Cowan et al., 2016) and pubertal timing disruptions in offspring (Cowan & Richardson, 2019). However, the studies included in this dissertation demonstrate that Lacidofil® does not have the same beneficial impact when the stressor is physiological, in the form of a Western diet. This suggests that probiotics must be tested not only for different applications but even within a specific type of application or use (i.e., for stress management) and that there may be sub-categories that require further research.

5.1.5 Minimal Overlap Between Probiotic and Diet Effects on Gut Microbiome

In my investigation of the gut microbiota of placebo-treated versus probiotic-treated rat dams and the combination of probiotic treatment and diet administration, I did not find that the probiotic Lacidofil® had a significant impact on the gut microbiome composition of these animals. Considering that the placebo and probiotic-only treated animals were otherwise healthy, it makes sense that no sweeping microbiome composition

changes were observed, as there would already be a stable microbiome in place in these animals (Moya & Ferrer, 2016). Therefore, large shifts based on probiotic administration were potentially impossible with a stable and functionally redundant microbiome present (Moya & Ferrer, 2016). Furthermore, there were few interacting effects when the probiotic was provided to the animals in combination with the Western diet. It is possible that the Western diet utilized for this study did not compromise the animals' health in a way that the probiotic could mitigate. Notably, this was not a study of any specific metabolic disease, such as non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH).

In preclinical animal studies of NAFLD or NASH, probiotic administration does interact with the disease state to have a greater impact on gut microbiome composition measures. For example, probiotic treatment with *L. rhamnosus* GG increased total bacteria numbers in HFD-fed animals compared to placebo-controlled HFD-fed animals (Ritze et al., 2014). The probiotic treatment of *L. plantarum* LC27, *B. longum* LC67, or their mixture reduced Proteobacteria levels and LPS production in HFD-fed mice (H. I. Kim et al., 2019). Researchers investigating a multi-strain probiotic treatment (five *Lactobacillus* and five *Enterococcus* strains) in aged (> 78 weeks) HFD-fed mice found that the probiotic increased alpha diversity and resulted in a unique beta diversity profile compared to placebo-treated controls (Ahmadi et al., 2020). Lastly, a mouse study administering an HFD for 16 weeks, with or without a multi-strain probiotic treatment (*B. subtilis* and *E. faecium*), found that the beta diversity of the gut microbiome was significantly impacted by probiotic treatment in conjunction with HFD (J. Jiang et al., 2021). Probiotics may have

a window of opportunity to impact the gut microbiome due to the severity of these diseases and the likelihood of a compromised gut microbiome.

5.1.6 Breast Milk Hormones Affected Separately by Treatment and Diet

Lastly, it is interesting that probiotic treatment with Lacidolfil® decreases leptin levels in breast milk. Although it did not help to lower the increased leptin levels found in milk from Western diet-fed dams, this finding suggests that probiotics should continue to be researched for how they impact breast milk composition. The studies conducted for this dissertation only collected breast milk at a single time point on one postpartum day. Future studies exploring more time points throughout the day and across multiple postpartum days may demonstrate more effects of probiotic treatment and interactions with maternal diet. It has been documented that milk composition, including fat, protein, and vitamins, changes over time (Nicholas & Hartmann, 1991).

OVERALL LIMITATIONS AND FUTURE DIRECTIONS

5.1.7 Western Diet Composition

Future research on the Western diet and its impacts on health outcomes must consider diet composition. For example, researchers must consider whether to investigate the presence or absence of specific ingredients (e.g., methionine and choline; (Larter et al., 2008) or model a whole dietary pattern (e.g., Western diet; (Lieber et al., 2004). In the case of modelling diseases related to obesity and diabetes mellitus, such as NASH, this disease can be induced through Western diet administration (Lieber et al., 2004) or methionine and choline deficiency model (Larter et al., 2008).

Since there are conflicting results on how maternal diet affects maternal care (Connor et al., 2012; Kougias et al., 2018; Purcell et al., 2011), future researchers may want to consider conducting a comparative study examining how different diets compositions (e.g., Western diet, high-carbohydrate diet, cafeteria diet) may impact these behaviours. Studies demonstrate that nutrition differentially impacts behaviour (anxiety-related behaviour: Myles et al., 2023, anxiety-like and depressive-like behaviour: Bonfim et al., 2021). This trend is also observed by researchers investigating the effects of commercially available Western diets on rodent maternal care behaviour (Connor et al., 2012; Kougias et al., 2018; Purcell et al., 2011).

Furthermore, as discussed in this dissertation, there are many links between maternal diet and postpartum depression (PPD; Baskin et al., 2015; Biaggi et al., 2016; Pina-Camacho et al., 2015). However, the rat model utilized in these studies is not a PPD model, as this type of animal modelling requires the demonstration of specific disease traits (Mir et al., 2022). Despite the complex nature of PPD, future preclinical studies should investigate these connections and how maternal diet impacts the mother to try to model smaller components of PPD in animals.

5.1.8 Facility Containment Levels and Specific Pathogen-Free Animals in Microbiome Studies

It must be noted that the studies included in this dissertation were performed in a containment level 1 laboratory facility. While strict, tested protocols were in place to ensure that the probiotic's cross-contamination risk was low (Myles, O'Leary, Romkey, et al., 2020), many materials (e.g., cages, bedding, water bottles) and the diets themselves could not be sterilized via autoclaving. Perhaps future studies will be able to determine how much

microbial “noise” is introduced to an animal model if these materials cannot be perfectly sterile. Also, the animals themselves, unless they are germ-free (i.e., without a microbiome), would have unique microbes when arriving at the facility. The studies within this dissertation all utilized specific pathogen-free (SPF) animals. Unlike germ-free animals, SPF rodents are not guaranteed to be free of all contamination but are bred to be devoid of select infectious pathogens (Dobson et al., 2019; Lane-Petter, 1962).

Regarding a translational research approach, I think it is perfectly fine to use SPF animals in microbiome research, as it is more representative of how people live (i.e., researchers will never be able to control the sterility of a person’s environment or what their microbiome profile is like when they begin a study). However, it would be interesting if future work could help sort through which microbes are present in the environment versus which microbes are affected by the experimental manipulation. Furthermore, although there are common pathogens that the major animal supply companies avoid in their SPF animals (e.g., *Salmonella spp.*, *Citrobacter rodentium*), this is not standard across companies (Dobson et al., 2019). Therefore, while using SPF animals is meant to decrease unwanted disease and variability in experimental results due to disease (Lane-Petter, 1962), there are still likely to be differences in microbes between different SPF animals.

5.1.9 Future Directions: Probiotics and Preventative Interventions for Maternal Health

Despite the knowledge that pregnancy complications affect the long-term health of offspring, there has been much less focus on how these complications affect the mother's long-term health (Rich-Edwards et al., 2014). More research is needed, as emerging evidence suggests that maternal health complications during pregnancy may increase

disease risk later in life (Callaway et al., 2007). Pregnancy complications such as preeclampsia, gestational diabetes mellitus (GDM; Fraser et al., 2012) and preterm birth (P. Wu et al., 2018) have been increasingly linked to the incidence of future cardiovascular disease (CVD). A systematic review of women who had experienced preeclampsia, compared to women without, found that after controlling for confounding variables, women with a history of preeclampsia were approximately twice as likely to experience cardiac disease as those unaffected (McDonald et al., 2008). Preeclampsia occurs in 3-5% of the population, and GDM occurs in 5-10% (H. L. Brown & Smith, 2020). Like preeclampsia, GDM increases the risk of diabetes (Kjos et al., 1995) and CVD (Goueslard et al., 2016).

Due to the future health implications of pregnancy complications, the study of maternal non-communicable diseases (NCDs), such as diabetes and CVD, has been recognized as an important research area by the International Federation of Gynecology and Obstetrics (Poon et al., 2018). The first trimester of pregnancy is ideally when preventative measures can be undertaken to reduce the risks of NCDs in the mother and her offspring (Poon et al., 2018). Lowering the risk of future NCDs in children can be helped by supporting healthy pregnancies (Poon et al., 2018). For example, screening for potential pregnancy complications to identify those at an increased risk of developing an NCD is an integral part of healthy pregnancy support.

Some of the identifiable high-risk factors for preeclampsia include chronic hypertensive disease, autoimmune disease, and pre-existing diabetes (Poon et al., 2018). Moderate-risk factors include mothers 40 years or older, BMI greater than 35 kg/m^2 at the first-trimester visit, first pregnancy or a family history of preeclampsia (Poon et al., 2018).

Furthermore, high maternal blood glucose levels add to the risk of developing adverse outcomes, such as preeclampsia and macrosomia (Wendland et al., 2012).

A meta-analysis from 30 different cohort studies found that GDM was a significant risk factor for the later development of type 2 diabetes (C. Song et al., 2018). Unfortunately, there is a significant lack of well-researched prevention strategies for this group (Gabbe et al., 2012).

Similar to GDM, preeclampsia and other hypertensive disorders of pregnancy can lead to future cardiovascular health issues in women, such as diabetes mellitus (Callaway et al., 2007) and cardiac disease (McDonald et al., 2008). Whereas preeclampsia can be screened for in the first trimester (conception to 12 weeks), GDM is currently diagnosed via maternal glucose challenge in the second trimester (24-28 weeks; (Poon et al., 2018). Presently, there is no early screening for GDM. Studies on preventative measures like dietary and physical activity interventions (Thangaratinam et al., 2012), probiotic use, (Brantsaeter et al., 2011; Luoto et al., 2010) and drugs like metformin (Syngelaki et al., 2016) are important.

5.1.10 Treatment Strategies for Gestational Diabetes, Preeclampsia and Hypertension

Due to the increased health risks associated with complications such as GDM and preeclampsia, the perinatal period is a good time to screen patients and provide interventions to improve long-term cardiovascular health (H. L. Brown & Smith, 2020).

Preclinical animal studies on metabolic disorders have found that probiotics and prebiotics may help treat obesity (Ejtahed et al., 2019), diabetes (Y. Wang, Dilidaxi, et al., 2020) and CVD (Cavallini et al., 2011). Similarly, studies have shown that synbiotics (i.e.,

a mixed formulation containing both a prebiotic and a probiotic) can improve biomarkers in coronary heart disease patients, including under the umbrella of CVD (Farrokhian et al., 2019; Tajabadi-Ebrahimi et al., 2017). Like other NCDs, CVD often induces a state of low-grade inflammation (H. Wu & Chiou, 2021). *Lactobacilli* strains, like those found in Lacidofil®, may help treat such diseases, as they help reduce inflammation (Brzozowski et al., 2006; Johnson-Henry et al., 2004). Additionally, probiotics' ability to modulate specific bacterial strains within the gut may be helpful (H. Wu & Chiou, 2021). For example, probiotics can increase beneficial bacteria such as *Prevotella* that produce anti-inflammatory metabolites (J. Li et al., 2016). A few suggested mechanisms for how probiotics confer these health benefits include epithelial barrier function, competition against pathogens, immune system modulation, and neurotransmitter and short-chain fatty acid (SCFA) production (C. Hill et al., 2014). In a mouse model, prebiotic intake through a high-fibre diet increased the number of bacteria in the gut capable of producing the SCFA acetate (Marques et al., 2017). Furthermore, the same high-fibre diet decreased systolic blood pressure in a hypertensive mouse model (Marques et al., 2017).

5.1.11 Gut Microbiota Profiling

The study of probiotics is relatively new despite a long history of human consumption (Ozen & Dinleyici, 2015; Sicard & Legras, 2011). Although the technologies for studying the microbiome (e.g., 16S rRNA sequencing) are becoming more affordable for researchers, there is still an ongoing debate regarding the best analysis practices (e.g., differential abundance analyses; Nearing et al., 2022; e.g., rarefaction; Schloss, 2024). Furthermore, bacterial metabolites must be examined to get a complete picture of what

these bacteria provide to the host. Metabolite-focused research would be beneficial in researching how probiotics may influence host behaviour, as I have found with maternal care behaviour. Additionally, investigating maternal neural pathways may provide further insight (e.g., investigating if probiotics affect the gene expression in brain regions involved in maternal behaviour neural circuitry).

Sequencing the gut microbiota profiles of different rodent strains (e.g., Long-Evans rats) is undoubtedly valuable information to share with other researchers, such that they can better characterize which microbes are typically found in their animal model compared to those they have found to be different in their studies. However, 16S rRNA gene sequence data is challenging to interpret without characterizing the potential functions or metabolites of the bacteria that are up or downregulated in response to experimental manipulation. Future research could investigate the metabolites or genetic pathways affected by Lacidofil® and the Western diet to understand better how these manipulations affect host physiology, utilizing tools compatible with 16S rRNA gene sequence data such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; Douglas et al., 2020). PICRUSt2 utilizes 16S rRNA sequencing data to predict metagenomes and their function (Douglas et al., 2020). Lastly, statistical power in microbiome research is also an issue (R. J. Moore & Stanley, 2016). Many animal studies work with small numbers, and while these numbers are sufficient for many behavioural and molecular outcomes, this does not appear to be true of microbiota analyses (R. J. Moore & Stanley, 2016).

Overall Interpretation

The studies included in this dissertation demonstrate that the probiotic Lacidofil® does impact some maternal and offspring outcomes but that this probiotic treatment does not target specific things affected by Western diet administration. This is evidenced by the main effects of probiotic treatment and the main effects of the Western diet, with little interaction between these experimental manipulations. This may again be a case for next-generation probiotics chosen for their ability to produce specific metabolites to confer benefits aimed towards mitigating specific health concerns.

Concluding Statement

When Barker and Osmond originally published their 1986 study, which would eventually give rise to the field of research known as the developmental origins of health and disease (DOHaD), it focused on how early insults *in utero* compromised the health of a generation. As such, the DOHaD hypothesis was initially called the fetal origins of health and disease hypothesis. Although the field of DOHaD research continues to grow and expand, there still seems to be a need for more research focusing on maternal health outcomes. If anything, I hope that the research in this thesis shows that maternal health should be further studied for its implications for the mother herself, in addition to offspring outcomes. My findings demonstrate that although probiotics have some effects on maternal behaviour, microbiota and offspring programming, maternal diet overwhelmingly influences health.

The perinatal period is a vulnerable time, and many unique diseases and disorders affect maternal health. Therefore, maternal health and wellness should be a primary focus

of DOHaD research. Pregnancy, although a natural occurrence, does bring the potential for many complications and health consequences, as discussed throughout this work. These complications not only affect the mother and her future health but may have lasting ramifications for the health of another generation. Furthermore, if researchers want to promote gut-brain health, this and other studies (Everard et al., 2014; Liang et al., 2021; Y. Liu et al., 2019; T. Wang et al., 2020; Q.-X. Zheng et al., 2021) demonstrate that diet is a huge factor in shaping the microbiome. For their part, probiotics and their applications must continue to be researched to determine the extent and therapeutic value of their effects.

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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.

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, %	28.50%
Fat (ether extract), %	13.49%
Carbohydrates, %	57.99%

*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.

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Product Data - D12079B

Description
RD Western Diet.

Used in Research
Obesity.
Diabetes.
Atherosclerosis.
Metabolic Syndrome.

Packaging
Product is packed in 2.5 kg foil-lined, heat-sealed bags within a 2.5, 5, and 12.5 kg box. Each box and bag is identified with the product name, description, lot number and expiration date.

Lead Time
Pelleted: in stock. 2-4 business days for 12.5 kg and increments.
5-7 business days for 2.5 and 5 kg increments.
Powder form: 5-7 business days.

Gamma-Irradiation
Yes. Add 5-7 business days to lead time.

Form
Pellet, Powder.

Shelf Life
Store in a cool and dry environment. Stored correctly diet should last 6 months.

Control Diets
Custom diets available on request.

Formula

Product # D12079B		gm%	kcal%
Protein		19.8	17
Carbohydrate		50.0	43
Fat		21.0	40
	Total		100
	kcal/gm	4.7	

Ingredient	gm	kcal
Casein, 30 Mesh	195	780
DL-Methionine	3	12
Corn Starch	50	200
Maltodextrin 10	100	400
Sucrose	341	1364
Cellulose	50	0
Milk Fat, Anhydrous*	200	1800
Corn Oil	10	90
Mineral Mix S10001	35	0
Calcium Carbonate	4	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
Cholesterol	1.5	0
Ethoxyquin	0.04	0
Total	**1001.54**	**4686**

*Anhydrous milk fat typically contains approximately 0.3% cholesterol. On this basis, D12079B contains approximately 0.21% cholesterol.
Formulated by E. A. Ulman, Ph.D., Research Diets, Inc., October 12, 1995.
Diet formulated to match Teklad Western Diet #TD88137, except that 1% Corn Oil replaces 1% Butter Fat.

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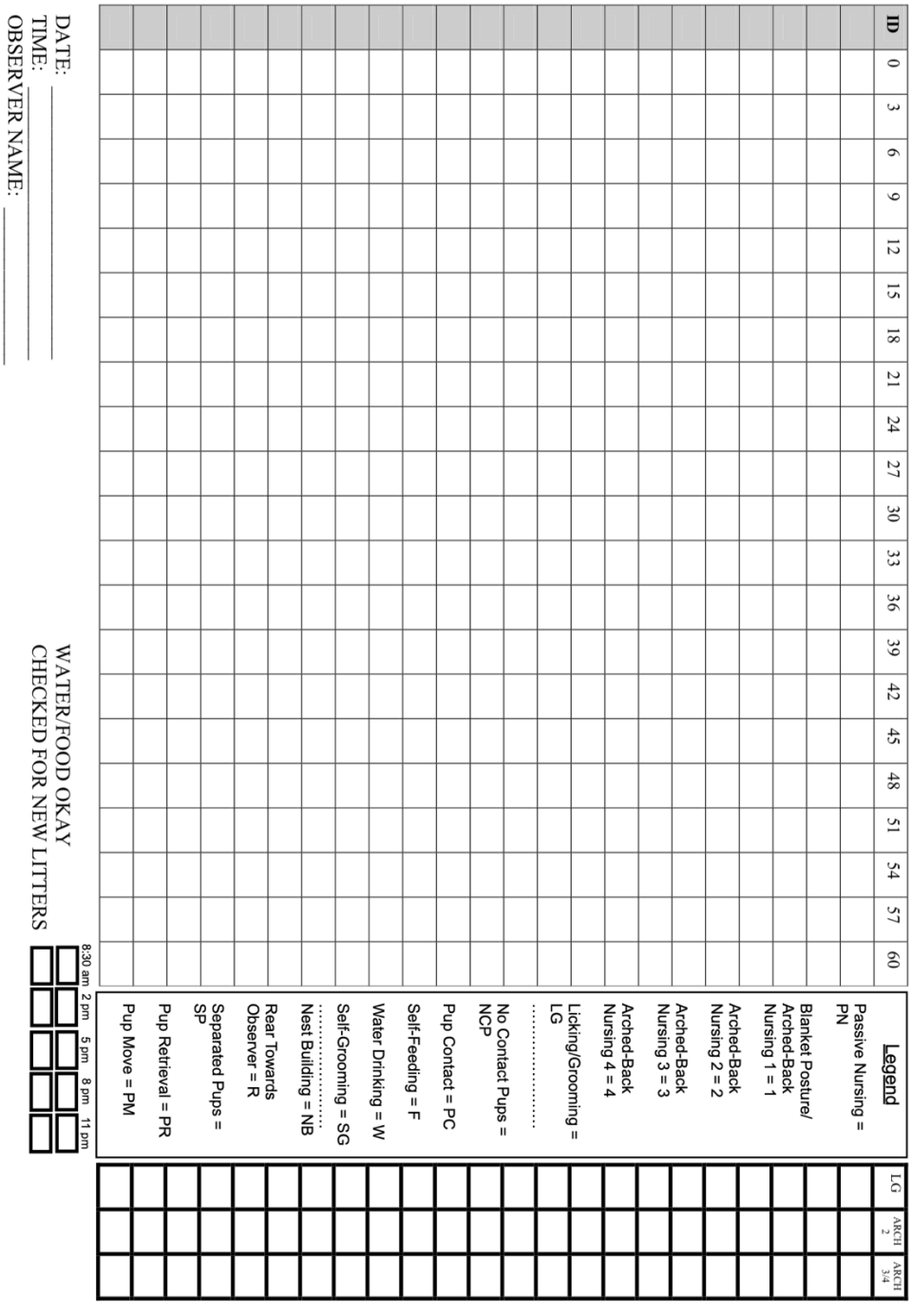
APPENDIX B MATERNAL CARE OBSERVATION RECORDING SHEET



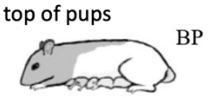
APPENDIX C MATERNAL CARE BEHAVIOURS ETHOGRAM

Maternal Care Behaviour Observations Ethogram

Perrot Lab (Adapted from Champagne et al., 2003[a] and Popoola et al., 2015[b])

Passive Nursing (PN): Dam lying on side nursing pups, but not in an arched back nursing posture

Blanket Posture (BP): Dam lying prone on top of pups



Arched-back nursing "2" (ABN 2): Dam's back slightly arched, legs slightly extended (but possibly not visible in nest)

Arched-back nursing "3" (ABN 3): Dam's back arched, legs extended, and visible beyond nest

Arched-back nursing "4" (ABN 4): Dam's back completely arched, legs fully extended, usually head is pointed down

Licking/grooming (LG): Dam engages in oral or anogenital licking of pups, can occur with arched-back nursing

Separated pups (SP): Away from the litter and/or outside the nest

Pup retrieval (PR): Carrying pups towards the nest

Pup move (PM): Carrying pups away from the nest

Pup contact (PC): Any contact with pups that does not include blanket-posture nursing, arched-back nursing or passive nursing

No contact with pups (NCP)

Feeding (F)

Nest building (NB)

Self-grooming (SG)

Rearing Towards Observer (R)

[a]Champagne, F. A., Francis, D. D., Mar, A., & Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiology & behavior*, *79*(3), 359-371.

[b]Popoola, D. O., Borrow, A. P., Sanders, J. E., Nizhnikov, M. E., & Cameron, N. M. (2015). Can low-level ethanol exposure during pregnancy influence maternal care? An investigation using two strains of rat across two generations. *Physiology & behavior*, *148*, 111-121.

APPENDIX D INFLAMMATORY ANALYTE FUNCTION, PATHWAY DESCRIPTIONS AND HUMAN ORTHOLOGS

Table 1 A list of the inflammatory analytes that were measured via multiplex ELISA assay in rat dams' plasma samples and found to have statistically significant differences between experimental groups, where Western diet-fed dams had higher levels of all inflammatory analytes. Each analyte has a description including their cellular pathway participation, their function in brief and their human gene ortholog.

Analyte	Cellular Pathway Participation (Smith et al., 2020)	Brief Description of RGD Function (Smith et al., 2020)	Human Gene Ortholog (Smith et al., 2020)
Interleukin-1β (IL-1β)	IL-1 and IL-12 signaling pathways; pro-inflammatory cytokine mediated pathway	Facilitates cytokine activity, involved in intracellular signal transduction	Human interleukin-1β (IL-1β)
Interleukin-7 (IL-7)	Cytokine mediated signaling pathway; Jak-Stat signaling pathway	Hypothesized to facilitate cytokine activity/participate in growth factor activity	Human interleukin 7 (IL7)
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)	Fc epsilon receptor mediated signaling pathway; Granulocyte -macrophage colony-stimulating factor signaling pathway; Syndecan signaling pathway	Facilitates cytokine activity, engages in cellular response to endotoxins	Colony-stimulating factor 2 (Csf2)
Growth Related Oncogene/Keratinocyte Chemoattractant (GRO/KC)	IL-23 signaling pathway; Chemokine mediated signaling pathway; NOD-like receptor signaling path	Facilitates chemokine activity, participates in neutrophil chemotaxis	C-X-C motif chemokine ligand 1 (CXCL1)
Monocyte Chemoattractant Protein (MCP-1)	Angiotensin II signaling pathway; Vascular endothelial growth factor signaling pathway; GM-CSF signaling pathway	Facilitates chemokine and heparin binding activity, involved with cellular responses to cytokines, lipids and leukocyte chemotaxis	C-C motif chemokine ligand 2 (Ccl2)
Macrophage Inflammatory Protein 1α (MIP-1α)	IL-12 signaling pathway; Chagas disease pathway; Chemokine mediated signaling pathway	Facilitates chemokine activity, participates in neutrophil chemotaxis	C-C motif chemokine ligand 3 (Ccl3)

[a]The analytes included in the multiplex ELISA assay that were not measurable in the plasma samples included: IFN-γ, IL-2, IL-4, IL-6, IL-12p70, IL-13, IL-17, M-CSF, TNF-α and VEGF.

[b]The analytes included in the multiplex ELISA assay that were measured but did not show any statistically significant differences between experimental groups included: G-CSF, IL-1α, IL-5, IL-10, IL-18, MIP-3α or RANTES.

APPENDIX E SAMPLE SPIKING CALCULATIONS

These are calculations for how much of each probiotic strain is required to spike each control sample to determine which sample will be used to generate a standard curve for strain detection.

***L. rhamnosus* R0011, Lot # 30VD0469:**

$$\frac{2.46 \times 10^{10}\ CFU/g}{1\ mL} \times \frac{10^{10}\ CFU}{x\ mL}$$

$$\frac{x\ mL = 1\ mL \times 10^{10}\ CFU}{2.46 \times 10^{10}\ CFU} = 0.4065\ mL\ of\ R0011\ solution\ (407\ \mu L)$$

***L. helveticus* R0052, Lot # JF5002:**

$$\frac{3.36 \times 10^{10}\ CFU/g}{1\ mL} \times \frac{10^{10}\ CFU}{x\ mL}$$

$$\frac{x\ mL = 1\ mL \times 10^{10}\ CFU}{3.36 \times 10^{10}\ CFU} = 0.2976\ mL\ of\ R0052\ solution\ (298\ \mu L)$$

APPENDIX F MASTER MIX CALCULATIONS

Table 1. Master Mix calculation table for qPCR strain detection.

Reagent	Volume (μL) per reaction	Number of Reactions[a]	Pipetting Error (× 1.1)	Final Calculated Volume (μL)
SYBR	12.5	20	1.1	275
Forward primer	0.075	20	1.1	1.65
Reverse primer	0.075	20	1.1	1.65
PCR-grade water	9.85	20	1.1	216.7

[a]This number can be adjusted, pending the number of reactions needed.

APPENDIX G 16S SEQUENCING-RELATED CALCULATIONS

Table 1. Master mix calculation table for amplicon PCR step used in microbiota profile sequencing.

Reagents	Starting Volume (µL)	Number of Samples	Pipetting Error	Final Volume (µL)
Microbial DNA (5 ng/µL)	2.5	31	1.1x	85.25
Amplicon PCR Forward Primer 1 µM	5	31		170.5
Amplicon PCR Reverse Primer 1 µM	5	31		170.5
2x KAPA HiFi HotStart ReadyMix	12.5	31		426.25
Total	25	31		852.5

Table 2. Master mix calculation table for indexing PCR step used in microbiota profile sequencing.

Reagents	Starting Volume (µL)	Number of Samples	Pipetting Error	Final Volume (µL)
Microbial DNA	5	31	1.1x	170.5
Index Primer 1	2.5	31		85.25
Index Primer 2	2.5	31		85.25
2x KAPA HiFi HotStart ReadyMix	12.5	31		426.25
PCR Water	5	31		170.5
Total	27.5	31		937.75

Table 3. Primer sequences for the 16S rRNA gene V3-V4 region.

Variable Region	Primer Name	Targeted Sequence	Amplicon Size (bp)	Annealing Temp (°C)
V3-V4	16S F	CCTACGGGNGGCWGCAG	444	55
	16S R	GACTACHVGGGTATCTAATCC		

APPENDIX H SOP FOR MILK COLLECTION

STANDARD OPERATING PROCEDURE	**No:** Perrot-2-2019
Authors: Elizabeth O'Leary, Libby Myles, Tara Perrot	**Version:** 3
	Revision Date: November 16, 2020
Postpartum Milk Collection Method for Long-Evans Rats	

1) PURPOSE

The purpose of this standard operating procedure (SOP) is to perform milk collection on postpartum female Long-Evans rats so that milk can be analyzed for bioactive compounds.

2) DEFINITIONS

a. Standard operating procedure – SOP
b. Oxytocin – OXT
c. i.p. – intraperitoneal
d. millilitre – mL
e. microlitre – μL

3) REFERENCES

DePeters, E. J., & Hovey, R. C. (2009). Methods for collecting milk from mice. *Journal of mammary gland biology and neoplasia*, *14*(4), 397-400.

Paul, H. A., Hallam, M. C., & Reimer, R. A. (2015). Milk Collection in the Rat Using Capillary Tubes and Estimation of Milk Fat Content by Creamatocrit. *JoVE (Journal of Visualized Experiments)*, (106), e53476.

Willingham, K., McNulty, E., Anderson, K., Hayes-Klug, J., Nalls, A., & Mathiason, C. (2014). Milk collection methods for mice and Reeves' muntjac deer. *JoVE (Journal of Visualized Experiments)*, (89), e51007.

4) RESPONSIBILTIES

4.1 It is the responsibility of the person(s) performing this protocol to read and apply this protocol properly when:
  4.1.1 Preparing the materials and equipment and
  4.1.2 When handling the animals and performing the procedure.
4.2 It is the responsibility of the person(s) performing this protocol to notify the lab manager immediately of any equipment malfunctions or issues.

5) MATERIALS

Table 1. All necessary materials for the milk collection procedure.

Name	Company	Catalogue No.	Notes	Status
1 mL syringes (sterile)	BD-Canada	309602	Lab 1252 – Labelled drawer	
26-gauge needles	BD-Canada	305122	Lab 1252 – Labelled drawer	
18-gauge needles	BD-Canada	305196	Lab 1252 – Labelled drawer	
Oxytocin (20 USP units/mL)	Vetoquinol	103644	Vet must order (from CDMV)	
Isoflurane Inhalation Anesthetic	-	-	In Psych surgery suite	
Sterile alcohol prep pads	WalMart	-	Lab 1252	

Absorbent bench pad	VWR	-	Lab 1252 – Under lab sink	
Heating pads	-	-	Lab 1252 – Labelled drawer	
Anesthesia machine	-	-	In Psych surgery suite	
Animal masks	-	-	In Psych surgery suite	
1.5 mL Microcentrifuge tubes	Axygen	MCT-150-C	At Dalhousie MedStores	
Professional trimmer	-	-	Lab 1252	
Surgical scissors	-	-	Lab 1252	
Eye lubricant	-	-	Surgery tech must order	
Ice bucket + ice	-	-	Lab 1252	
P200 pipette	-	-	Lab 1252	
P200 sterile filtered tips	VWR	89140-936	Lab 1252	
Prevail® Laboratory Disinfectant	Virox	02436809	Animal Care	

6) PROCEDURE

SEPARATE DAM FROM OFFSPRING

6.1 Separate the dam(s) to be milked from her offspring, for a **MINIMUM** of 5 minutes prior to milking (Paul et al., 2015). The dam should be placed in a fresh cage, with food and water available.

Note. Willingham et al., 2014 and DePeters & Hovey, 2009 suggest that separating the dam for a 2-hour period prior to milking is ideal. Also, Paul and colleagues (2015) note that periods of separation of greater than 4 hours may alter milk composition.

6.2 The offspring cage should be placed on a heating pad, set at a medium heat setting, while the dam is being milked.

PREPARE MATERIALS FOR MILK COLLECTION

6.3 Collect the required materials for the milking procedure, as outlined in Table 1 (see Section 5).

6.4 Disinfect the area to be used for the procedure with Prevail® laboratory disinfectant spray or wipes.

6.5 Use a heating pad, set at medium heat, with absorbent bench pad overtop, or a heated surgical table to maintain the dam's body temperature during anesthesia.

6.6 Set-up the anesthesia system within reach of the experimenter, including the anesthetic mask/cone for the animal.

6.7 Using an aseptic technique, set-up a 26-gauge needle with a 1 millilitre (mL) syringe to be used for OXT injection.

ANESTHETIZATION

6.8 Open the oxygen tank and turn the flow to 1 L (1,000 cc) per minute.

6.9 Turn on the flow of isoflurane and set it to 5%.

6.10 Anesthetize the dam, and place her on her back, on the heating pad/surgical table.

6.11 Confirm that the dam is sufficiently anesthetized by checking for a pedal reflex.

6.12 Reduce the isoflurane flow to 2-3% to maintain anesthesia and monitor the dam throughout the procedure.

6.13 An eye lubricant should be applied to the corners of the dam's eyes to ensure the eyes are protected from drying out and/or scratches.

OXYTOCIN INJECTION

6.14 The vial of OXT should be disinfected with a sterile alcohol pad.

6.15 Draw up 2 IU (0.1 mL) of OXT into the syringe. This dose can be repeated **ONCE** if needed to continue milk letdown.

Note. Alternatively, a dose of 4 IU/kg body weight can be used.

6.16 OXT should be injected intraperitoneally (i.p.), with the needle injected in the lower right quadrant of the abdomen, 0.5 cm deep, at an angle of 15-30^{o}.

6.17 Wait approximately 1-15 minutes for the OXT to stimulate milk letdown.

PREPARATION OF MILK SITES

6.18 Gently use trimmers to remove hair around the mammary gland(s) to be milked.
6.19 Sterilize mammary gland(s) using a sterile alcohol pad.

MILK COLLECTION

6.20 The milk letdown should become noticeable in the mammary gland.
6.21 Using the thumb and forefinger to manually squeeze the gland in an upward direction until a bead of milk forms.

Note. If the milk will be used for 16S bacteria microbiota profiling, the first few drops of milk should be discarded, as it is possible that they will be contaminated by the dam's skin.

6.22 With a 50 μL micro-dispenser capillary tube, touch the top of the bead of milk.
6.23 Expel the milk sample into a microtube using a syringe to displace from the capillary tube, and place on ice.
6.24 Repeat steps 6.20-6.23 on the next mammary gland.
6.25 When the procedure is complete, the oxygen and isoflurane flow should be switched **OFF**.
6.26 Place milk samples in a labelled box, in the appropriate drawer in the -80^{o} freezer.

ANESTHETIC REVERSAL

6.27 For recovery, the dam should be placed in a cage lined with absorbent bench pad (cage bedding might scratch the eyes).
6.28 Dam should be monitored until they have regained consciousness and are able to move around the cage without struggling.
6.29 Once the dam has recovered, she can be returned to her pups.

7) DOCUMENT HISTORY

Table 2. List of SOP versions, description of changes, reviewers and dates of revision.

VERSION	DESCRIPTION OF CHANGES	REVIEWER	DATE
1	SOP was created		April 10, 2019
2	SOP was edited – including an updated list of materials and an added step for potential milk collection (multiple methods will be tried to determine the optimal sample collection method).	Elizabeth O'Leary	July 2, 2019
3	SOP was edited – best milk collection method from last experiment (ethics approval #19-028) was determined to be microcapillary tube – other methods were deleted.	Elizabeth O'Leary	November 16, 2020

APPENDIX I PRIMER INFORMATION FOR RT-QPCR EXPERIMENTS

Table 1 Primer IDs, product sizes (in base pairs; bp), melt temperatures in degrees Celsius (°C) and the forward and reverse sequences for the RT-qPCR experiment analyzing offspring hippocampal gene expression.

Primer	Product Size (bp)	Melt Temp (°C)	Forward Primer	Reverse Primer
RAT_NPY[1]	288	60	5′–GCTAGGTAACAAACGAATGGGG–3′	5′–CACATGGAAGGGTCTTCAAGC–3′
RAT_GAPDH[1]	184	60	5′–CAGTGCCAGCCTCGTCTCATA–3′	5′–TGCCGTGGGTAGAGTCATA–3′
RAT_RPL13A[2]	132	60	5′–GGATCCCTCCACCCTATGACA–3′	5′–CTGGTACTTCCACCCGACCTC–3′
RAT_rGR3	188	60	5′–GCTTCAGGATGTCATTACGGGG–3′	5′–GCTTCAAGGTTCATTCCAGCC–3′
Oligo DTs	–	–	5′–TTTTTTTTTTTTTTTTTTTTTTTV N–3′	–

[1]Shi et al., 2009

[2]Langnaese, John, Schweizer, Ebmeyer, & Keilhoff, 2008

[3]Mashoodh, Sinal, & Perrot-Sinal, 2009