MATERNAL MICROBES: THE EFFECTS OF PROBIOTIC TREATMENT ON MATERNAL CARE BEHAVIOUR, INFLAMMATION AND OFFSPRING STRESS OUTCOMES

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
July 2019

DEDICATION PAGE

For my parents, Joe and Shelley O'Leary. You both taught me the importance of hard work and dedication.

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ABSTRACT

Active maternal care is vital in offspring stress programming, yet the behaviour of a mother towards her offspring can also be influenced by her external environment. It is essential to understand what impacts maternal care, as poor maternal care can disrupt offspring development, which can affect later health outcomes. Probiotics have the potential to alter the availability of hormones, (e.g., oxytocin and estrogen), which initiate and maintain maternal care behaviour. We investigated the effects of a probiotic treatment, Lacidofil®, on maternal care, inflammation and offspring stress-related outcomes. Overall, we found that probiotic-treated mothers performed increased amounts of active nursing and licking/grooming, compared to their placebo-treated counterparts. Offspring from probiotic mothers weighed significantly more than offspring from placebo-treated mothers at weaning. We observed a few trending differences in offspring stress-related outcomes. These results contribute to our understanding of the external factors that can influence maternal behaviours and ultimately affect offspring outcomes.

LIST OF ABBREVIATIONS AND SYMBOLS USED

UNITS

%	Percentage
°C	Degrees Celsius
Cq	Quantification cycle value
cm	centimetre
mm	millimeter
M	Molar
mM	Millimolar
µM	Micromolar
g	Gram
mg	Milligram
kg	Kilogram
µg/ng/pg	Micro/nano/picogram
L	Litre
mL	Millilitre
µL	Microlitre
CFU	Colony Forming Units
W	Watt
RPM	Revolutions per minute
g	Standard gravity
m/s	Meters per second
h	Hour

MOLECULAR TERMINOLOGY

qPCR	quantitative Polymerase Chain Reaction
gDNA	Genomic Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl Fluoride
DMS	Dimethyl Sulfide

DISCIPLINE-SPECIFIC ACRONYMS

HPA	Hypothalamic-Pituitary-Adrenal
SNS	Sympathetic Nervous System
PVN	Paraventricular Nucleus

DISCIPLINE-SPECIFIC ACRONYMS (CONT'D)

CRF	Corticotropin-Releasing Factor
ACTH	Adrenocorticotropic Hormone
CORT	Corticosterone
ABN	Arched-Back Nursing
LG	Licking/Grooming
GR	Glucocorticoid Receptor
MR	Mineralocorticoid Receptor
SNH	Semi-Naturalistic Housing
IBD	Irritable Bowel Disease
mPOA	Medial Preoptic Area
OTR	Oxytocin Receptor
ER-α	Estrogen Receptor Alpha
GF	Germ-Free
SPF	Specific Pathogen-Free
FST	Forced Swim Test
EPM	Elevated Plus Maze
OFT	Open Field Test
BLA	Basolateral Amygdala
ELS	Early Life Stress
PVC	Polyvinyl Chloride
GD	Gestational Day x
Px	Postnatal Day X
PPDx	Postpartum Day X
CCAC	Canadian Council on Animal Care
UCLA	University Committee on Laboratory Animals
BSA	Bovine Serum Albumin
VP	Vasopressin
L.	Lactobacillus
B.	Bifidobacterium

INFLAMMATORY MARKERS

IL-x	Interleukin-x
NF-κβ	Nuclear Factor kappa beta
GM-CSF	Granulocyte/Macrophage-CSF
M-CSF	Macrophage-CSF
GRO/KC	Growth Related Oncogene/ Keratinocyte Chemoattractant
RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted

INFLAMMATORY MARKERS (CONT'D)

MIP-1α	Macrophage Inflammatory Protein-1 alpha
MIP-3α	Macrophage Inflammatory Protein-3 alpha
MCP-1	Monocyte Chemoattractant Protein-1
IFN-γ	Interferon gamma
TNF-α	Tumor Necrosis Factor alpha
G-CSF	Granulocyte-Colony Stimulating Factor
VEGF	Vascular Endothelial Growth Factor

SYMBOLS

®	Registered Trademark Symbol

ACKNOWLEDGEMENTS

I acknowledge and give many thanks to my supervisor, Dr. Tara Perrot, for her assistance, expertise and unwavering support throughout completing my thesis. Thank you to my thesis committee members, Drs. Shelley Adamo and Tamara Franklin, for your insights in the making of this project. This work was supported by an Engage Grant from the Natural Sciences and Engineering Research Council (NSERC), awarded to Dr. Tara Perrot. I am grateful to have been supported by a Mitacs Accelerate Fellowship and a Nova Scotia Graduate Scholarship (NSGS). Thank you to our industry collaborator, Dr. Thomas Tompkins and his team at the Rosell Institute for Microbiome and Probiotics, the Research and Development group of Lallemand Health Solutions, Inc. I acknowledge my fellow graduate student, Libby Myles, for her help in running the experiments included herein, and for her support and companionship throughout the last two years. Thank you to all the students and volunteers from Dr. Perrot's laboratory for your assistance with this project. Thank you to my partner, Alex Walker. Without his continued love and support, I would not have gotten this far, or at the very least, I would be in far worse shape. Thank you to my family for always being a source of love and support, and my friends for providing love, food, and wonderful company.

CHAPTER 1 INTRODUCTION

The development of mammals, including rats, is heavily influenced by both the prenatal and postnatal environments. Both during gestation and the postpartum period, how the mother is affected by her internal and external states, in turn, affects the programming of her offspring's neural, physiological and behavioural responses to stress.

The programming of the hypothalamic-pituitary-adrenal (HPA) axis, the primary stress-response circuit, is a prime example of this programming as it has previously been shown to be affected by prenatal events, such as prenatal stress, and by the postnatal context, including the quantity and quality of maternal care provided to the offspring. Since the mother is the bridge between her offspring and their environment, understanding when and how the environment can impact the mother provides a potential means for influencing offspring programming while also giving valuable insight into the mechanisms by which systems, such as the HPA axis, develop in mammals.

1.1 THE STRESS RESPONSE

The simplest definition of stress is that it is a situation that endangers an individual's homeostasis (Selye, 1956; Smith & Vale, 2006). A stressor is an event that stimulates the beginning of the stress state, and as such, can include (real) physical, or (perceived) psychological threats to an organism's life. To manage the imbalance created by the stressful event, endocrine, nervous and immune systems mount a response to restore, and regulate bodily functions (Smith & Vale, 2006). When an organism is presented with a stressor, this triggers the classic fight-or-flight response (Schwabe et al., 2012; Finsterwald & Alberini, 2014). The fight-or-flight response prompts adrenergic

transmission within the sympathetic nervous system (SNS), which activates the HPA axis (Smith & Vale, 2006; Finsterwald & Alberini, 2014). The HPA axis is the primary stress response circuit in mammals and is comprised of the hypothalamus, the pituitary gland and the adrenal glands. As a result of the connectivity between the amygdala and the hypothalamus, when a stressful event activates the amygdala, the paraventricular nucleus (PVN) of the hypothalamus secretes corticotropin-releasing factor (CRF) and vasopressin (VP; Smith & Vale, 2006; Hill & Tasker, 2012). The release of CRF and VP from the hypothalamus into pituitary portal circulation triggers the anterior lobe of the pituitary gland to release adrenocorticotropic hormone (ACTH; Finsterwald & Alberini, 2014; Hill & Tasker, 2012; Smith & Vale, 2006). When ACTH is released into the circulatory system, this stimulates the adrenal cortex, which then begins to synthesize and secrete adrenaline, noradrenaline and glucocorticoids such as corticosterone (CORT), into circulation (Finsterwald & Alberini, 2014; Hill & Tasker, 2012; Smith & Vale, 2006). The initial release of both adrenaline and noradrenaline prepares the animal for the stressful event by elevating its heart rate, increasing its blood circulation to its muscles, and the commencement of glucocorticoid synthesis in the adrenals (Finsterwald & Alberini, 2014; Hill & Tasker, 2012; Smith & Vale, 2006). These consequences of the initiation of the stress response allow mammals to adapt appropriately to the present situation so that they can survive (Weschler, 1995; Hawley et al., 2010). Glucocorticoids then help to restore homeostasis and allow for adaptation post-stressor, by acting on the metabolic, cardiovascular, immune, and behavioural processes of an organism (De Kloet et al., 1998; Finsterwald & Alberini, 2014; Hill & Tasker, 2012; Smith & Vale, 2006). Importantly, glucocorticoids also end the HPA axis response through negative feedback

control (De Kloet et al., 1998). This negative feedback loop is completed when the secreted glucocorticoids bind to glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) in the hypothalamus, pituitary gland and hippocampus, leading to a dampening of future CRF release (Cole et al., 2000; De Kloet et al., 1998; Evanson et al., 2010; McEwen, De Kloet & Rostene, 1986; Sapolsky et al., 1984). MRs show high affinity for both aldosterone, the principal mineralocorticoid hormone, and corticosterone in rats, whereas GRs have lower affinity for corticosterone binding (McEwen, De Kloet & Rostene, 1986; Reul & De Kloet, 1985). Under normal conditions (i.e., with no stressor present), glucocorticoids bind to MRs (McEwen, De Kloet & Rostene, 1986). However, when an animal is faced with a stressful event, and there is a resulting increase in glucocorticoid levels, both MRs and GRs are bound and activated (Reul & De Kloet, 1985).

In addition, there are hormones, such as melatonin, that work in opposition to stress hormones such as CORT (Maestroni, Conti, & Pierpaoli, 1987). The hormone melatonin, produced by the pineal gland, is also affected by stress, and has immunomodulatory effects on natural killer cells, neutrophils, T cells and macrophages (Garcia-Maurino, Pozo, Carrillo-Vico, Calvo, & Guerrero, 1999). Due to these regulatory effects, melatonin also alters cytokine secretion and can act in direct opposition to acute CORT release (Maestroni, Conti, & Pierpaoli, 1987; Guerrero & Reiter, 2002).

Cytokine secretion, in response to a stressor, occurs through activation of the immune system via the HPA axis' initial response to the event in question (Bosch, 2005).

Cytokines are secreted proteins that participate in a cascade-like fashion in the expression of inflammatory reactions (Zhang & An, 2007). There are a few different types of cytokines, depending on the type of cell that synthesizes them and its cellular properties (Zhang & An, 2007). A few examples of cytokines include chemokines, cytokines with chemotactic abilities, monokines, cytokines produced by monocytes, and interleukins, cytokines made by leukocytes, that act on other leukocytes (Zhang & An, 2007). Although there is considerable research that suggests that stress suppresses immune function, including cytokine expression, this response may depend mainly on the nature of the stressor (Cohen, Tyrrell & Smith, 1991; Dhabhar & McEwen, 1997; Maier, Watkins & Fleshner, 1994). Some acute stressors, including predator threat, may provoke an increased immune response, to prepare the animal for the predator's attack, and any subsequent injury (Dhabhar & McEwen, 1997). Inflammation occurs when there are considerable alterations in the plasma concentration of acute-phase proteins, such as C-reactive protein, that mount the "acute-phase response" to inflammation (Gabay & Kushner, 1999). This response includes the activation of toll-like receptors (TLRs), which go on to activate the nuclear factor NF-κB pathway, an inflammation-inducing cellular pathway, inducing cytokine production (Gabay & Kushner, 1999; Tian et al., 2014). There are moderate, detectable changes in the acute-phase response after intense exercise or parturition, with smaller changes noted after exposure to psychological stress (Gabay & Kushner, 1999; Maes et al., 1997). Acute mental stress can induce the immune response through its ability to alter the distribution of peripherally circulating leukocytes (Bosch, 2005). There is also evidence to suggest the contrary, that acute stress, due to the increase in CORT levels, may inhibit the NF-κB pathway, and the resulting cytokine

production (Kadmiel & Cidlowski, 2013). Thus, it remains unclear how inflammation may arise in situations of acute stressors, and previous research suggests that the immune response may vary based mainly on the type of stressor encountered (Breen et al., 2016; Dhabhar & McEwen, 1997; Gabay & Kushner, 1999).

Physiological and behavioural changes associated with the stress response are vital to survival, and as such, are fine-tuned during early life in response to the present context/environment (McLeod, Sinal & Perrot-Sinal, 2007). Thus, the early life environment of mammals serves to program stress responding and stress resiliency, which persists throughout development into adult life (Caldji, Diorio & Meaney, 2000). For many mammals, the mother is the conduit through which the early environment is experienced by the developing offspring. It's not surprising, then, that a critical factor in the early life programming of the HPA axis and the stress response as a whole, is the care provided to animals by the mother (Meaney & Szyf, 2005). Thus, any alterations of the mother's behaviour, whether through prenatal/postnatal stress, nutrition, environment, can have profound effects on the offspring.

1.2 MATERNAL CARE

The mechanism by which early life events, particularly those in the first few weeks of life, permanently shape the brain and behaviour of offspring involves the actions of the dam toward her offspring, known as maternal care, comprised of well-characterized behaviours.

Maternal care is a vital component of the postnatal environment of mammalian species, including rats, monkeys and humans (Champagne et al., 2001, Bailey et al., 1999, Egeland & Hiester, 1995). In rats, dams provide their offspring with the basic needs required for survival, including milk, shelter, and grooming (Kristal, 2009). However, even in delivering these immediate needs, dams can influence their offspring's development beyond the postnatal period, and into adolescence and adulthood (Meaney & Szyf, 2005; Masis-Calvo et al., 2013).

In rats, natural variations in maternal care have been shown to affect the development of offspring fear responses, cognition, and stress responses (Champagne et al., 2003a; Liu et al., 2000). When a dam nurses her pups, she gathers them into her nest and stands over the nest to maximize the number of pups she can nurse at one time, a behaviour known as arched-back nursing (ABN; Kristal, 2009). At the same time as the dam nurses, she often licks and grooms (LG) her pups to stimulate the urogenital area and clear away the resulting waste (Kristal, 2009). Without this stimulation from the dam, her altricial offspring are unable to eliminate waste on their own (Kristal, 2009). This behaviour is also of benefit to the dam, as the waste contains water and electrolytes, which are depleted during parturition (Kristal, 2009). It is these two behaviours, arched-back nursing and licking/grooming that have a particularly meaningful connection to offspring development (Francis & Meaney, 1999; Champagne et al., 2003a). It is also these two behaviours that vary naturally across female rat populations (Champagne et al., 2003a). Offspring of mothers that perform high levels of LG/ABN have specific effects on behavioural outcomes, as well as the stress response, and brain morphology (Champagne

et al., 2003a; Champagne et al., 2008; Zhang et al., 2005). For example, female offspring of high LG/ABN dams demonstrate high levels of LG/ABN when they have their own litters (Champagne et al., 2003a). The stress-induced transmission of dopamine in the medial prefrontal cortex (mPFC) is lower in offspring from high LG/ABN dams, compared to offspring from low LG/ABN dams (Zhang et al., 2005). High LG/ABN offspring also have improved long term potentiation (LTP) in baseline conditions, whereas their low LG/ABN counterparts have poor LTP in baseline conditions, accompanied by shorter dendrite branches and low dendritic spine density in area CA1 of the hippocampus. Previous research has shown that offspring who have been exposed to high levels of maternal LG/ABN have reduced ACTH and CORT levels post-stressor (Liu et al., 1997). This more modest HPA axis response in offspring of high LG/ABN dams is due to the ability of these maternal care behaviours to increase the sensitivity of the HPA axis' negative feedback system (Meaney & Szyf, 2005; Korosi et al., 2010). It has been shown that pups raised by dams that engage in higher levels of LG have increased numbers of hippocampal glucocorticoid receptors (GRs), and thus, show more robust negative feedback regulation of the HPA axis (Meaney & Szyf, 2005). LG behaviour also promotes lower levels of CRF, resulting in the lower levels of ACTH and CORT that are observed (Liu et al., 1997; Meaney & Szyf, 2005; Korosi et al., 2010). Similarly, it has been found that the connectivity of CRF neurons is altered with offspring that received enhanced maternal care (performed by a brief separation protocol) having fewer vGlut2-immunoreactive boutons in contact with CRF neurons, reducing CRF activity (Korosi et al., 2010).

The differences observed in the levels of LG/ABN provided to offspring are most noticeable throughout the first seven to eight days of life (Champagne et al., 2003a). However, the quality of maternal care is independent of litter size and it is not related to pup killing, weaning survival or weaning weight (Champagne et al., 2003a; Meaney & Szyf, 2005). If the litter size is not a factor in the maternal care quality, we must consider other elements within the dam's external (e.g., level of predation threat; McLeod et al., 2007) and internal environments (e.g., nutritional status; Curley et al., 2011).

The maternal mediation hypothesis proposes that the outcome of any postnatal manipulation (e.g., early handling, maternal separation, enhanced environment) is mediated through alterations in the dam's behaviour towards her offspring (Claessens et al., 2011). Indeed, as mentioned previously, there have been many different types of manipulations used to alter maternal care behaviour to elucidate the mechanisms that influence this behaviour, including pharmacological treatment, lesions and targeted gene knockout (Champagne et al., 2004). In one of the first examples of maternal mediation in rats, Levine (1957) found that one way of inducing changes in offspring stress reactivity was to handle neonatal pups daily from birth to weaning (a total of 21 days). The handling paradigm resulted in brief periods of maternal separation and induced a hypo-responsiveness of the HPA axis in the handled offspring, including lower emotionality and improved cognition when compared to their non-handled counterparts (Levine, 1957). It was later found that the brief periods of maternal separation induced by Levine's daily handling protocol resulted in increased levels of maternal behaviour upon reuniting the mother and her offspring (Liu et al., 1997).

Other work has shown that changes to the dam's environment influence her maternal behaviour. A study by McLeod and colleagues (2007) demonstrated that an acute exposure to a predator odour stressor shortly after parturition increased both active maternal care behaviours, LG and ABN. This finding was replicated in the same laboratory in another study that demonstrated that the effects of predator odour exposure on maternal care after parturition are time-sensitive (Mashoodh, Sinal & Perrot-Sinal, 2009). Predator odour exposure only increased maternal LG/ABN if the exposure occurred within a few hours of birth (Mashoodh, Sinal & Perrot-Sinal, 2009). More recently, work performed by Korgan and colleagues (2018) demonstrated that the type of environment a dam lives in (standard cage versus a semi-naturalistic setting with a burrow), also influences maternal care, such that dams in semi-naturalistic housing (SNH) show increased levels of LG/ABN towards their offspring. These studies demonstrate that the surrounding environmental conditions that a dam is exposed to have powerful programming effects on the dam, as they foreshadow what her offspring may need to be prepared to encounter in their lifetime (Mashoodh, Sinal & Perrot-Sinal, 2009; McLeod, Sinal & Perrot-Sinal, 2007).

Thus, it is clear that features of the external environment (e.g., predation threat level, complexity) can be transmitted to offspring via changes in maternal care. It is not surprising, then, that features that signal the nutritional capacity of the environment, may also impact maternal care, thereby being transmitted to offspring. This is reflected in a body of work devoted to understanding how maternal diet may influence postnatal maternal behaviour, and whether or not this affects the offspring's behavioural phenotype

later in life (Connor et al., 2012). For example, it was found that dams provided a high-fat diet throughout pregnancy had reduced levels of maternal care (Connor et al., 2012). These results suggest that maternal nutrition influences maternal behaviour. However, the mechanism by which maternal nutrition could affect maternal care remains unknown. Collectively, these studies demonstrate that the programming of offspring in early life is subject to the immense sensitivity of the mother to not only her surrounding environment but her internal state as well.

1.3 THE MICROBIOME, GUT-BRAIN AXIS, AND PROBIOTICS

The mammalian microbiome has a diverse array of microorganisms, including bacteria, archaea, fungi and viruses, that inhabit both the body's interior and exterior (Sharon, Sampson, Geschwind & Mazmanian, 2016). The colonization of the microbiome begins at birth, where the vaginal microbiome is transferred from mother to offspring (Dominguez-Bello et al., 2010; Huure et al., 2008; Asnicar, 2017). With this colonization, infants are shown to develop microorganisms in their gut such as Bifidobacterium and Bacteroides (Jasarevic, Rodgers, & Bale, 2015), with the gut microbiota in mammals estimated to consist of greater than 40,000 species of bacteria (Sudo et al., 2012). These microorganisms contribute to daily bodily functions including immune function, digestion, nutrient absorption, and neurotransmitter modulation (Gareau, Sherman & Walker, 2010; Jasarevic, Rodgers, & Bale, 2015).

An increasing amount of research over the past decade has focused on the microbiome and its many functions, because research studies have demonstrated that the microbiome can be dysregulated, such that it is unable to operate optimally. This has been shown in

studies of functional bowel disorders, such as irritable bowel disease (IBD), where there is an abundance of Proteobacteria, a phylum of bacteria that contains *Escherichia coli*, which is associated with human disease states (Mukhopadhya, Hansen, El-Omar & Hold, 2012). This dysregulation of the microbiome, known as dysbiosis, has led to questions of what makes a microbiome "healthy." The features of a healthy microbiome include resistance, resilience and functional redundancy, as well as the absence of overt disease (Moya & Ferrer, 2016; Lloyd-Price et al., 2016). In particular, functional redundancy is an essential feature of the microbiome, and this is observed in highly diverse microbiomes (Lloyd-Price et al., 2016). Functional redundancy is a key attribute, as it can contribute to temporal stability of the microbiome, which is essential, as the relative amounts of a particular microbe can change in response to life events (Moya & Ferrer, 2016; Lloyd-Price et al., 2016). Pregnancy is a life event where the gut microbiota profiles of women have been observed to shift across trimesters in a fashion that cannot be predicted by diet or health status (Koren et al., 2012). A study by Koren and colleagues (2012) found that women in their third trimester had increased amounts of Proteobacteria in their stool as well as low-grade inflammation. This late pregnancy dysbiosis could also harm the offspring, as not only is the infant gut microbiome colonized through parturition, but breast milk also contains microbes, including *Lactobacillus* and *Bifidobacterium* strains (Fernandez et al., 2013). Lactobacilli and Bifidobacteria are present in human breast milk and not found in swabs of breast tissue from the same women (Gueimonde et al., 2007; Martin et al., 2009). Furthermore, Bifidobacteria are anaerobic bacteria, and thus it is highly unlikely that it would inhabit the mother's skin (Gueimonde et al., 2007). Together, these findings suggest that there is

an endogenous pathway for the transfer of maternal microbes to breast milk (Fernandez et al., 2013). One possibility for this endogenous pathway is through dendritic cell transport (Rescigno et al., 2001). Dendritic cells penetrate the gut epithelium, where they can transport non-pathogenic bacteria (e.g., probiotics) through the tight-junctions of cells, without disrupting gut epithelial integrity (Rescigno et al., 2001). Once the bacteria are carried out of the gut lumen, it is possible that they circulate through the body via lymphocytes, which are also found in breast tissue (Ferguson, 1985; Fernandez et al., 2013; Rescigno et al., 2001).

To help with the health of mothers and their offspring, it has been suggested that the gut microbiome of mothers be supplemented with probiotics, as they may have the ability to program the offspring's commensal microbiota using strains that have been associated with positive health benefits (Asnicar et al., 2017). Gestation and early postnatal periods are crucial for the formation of a healthy microbiome (Lloyd-Prince et al., 2016; Yatsunenko et al., 2012) and since bacteria can be transferred from mother to offspring, this may allow for the transfer of specific probiotic strains with demonstrable benefits to be supplemented into the offspring's microbiome (Dotterud et al., 2015). Providing additional microbes to augment the microbial population could perhaps increase the diversity of the microbe population, which is associated with health and functional redundancy (Moya & Ferrer, 2016). It has also been demonstrated in animal models that microbes given to the mother are detectable in her breast milk, implying that it is possible for these microbes to be transferred throughout nursing (Cowan, Callaghan & Richardson, 2016).

Probiotics are live microorganisms, typically bacteria or yeast, that when provided to the host in sufficient amounts, will confer a health benefit (Sanders, 2009). Probiotics must be provided to the host as live microbes, and the product must be able to survive the digestion process (i.e., stomach acid and bile; Sanders, 2009; Ljungh & Wadstrom, 2006). The genus of probiotic bacteria, *Lactobacillus*, is a popular probiotic supplement, due to many studies that have demonstrated the benefits of various strains of *Lactobacillus*, including the secretion of microbicidal substances, the modulation of enzymes, and deconjugation of bile acids (Ljungh & Wadstrom, 2006). These functions all contribute to the improved integrity of the gut lining, and overall host health (Ljungh & Wadstrom, 2006).

There is a growing body of research that demonstrates the importance of the "gut-brain axis," the bi-directional connections that exist between neural circuitry and gut physiology, on stress-responsive neural pathways, such as the HPA axis (Ait-Belgnaoui et al., 2012; Foster, Rinaman, & Cryan, 2017). The intestinal tract and the microbes that inhabit it are essential for the digestion of food, absorption of nutrients, and the production of neurotransmitters, including serotonin and dopamine (Sarkar et al., 2016). Due to the gut's connection to stress-responsive pathways such as the HPA axis, stressful events can negatively impact the host's gut health (Sarkar et al., 2016). For example, CORT secretion in response to stress harms the gut through the disruption of gut barrier function and increased intestinal permeability (Sarkar et al., 2016).

The consequences of disrupted gut function, and the therapeutic potential of probiotics are modelled in animals, including rodents, using different early life stressors. In these animal models of early life stress, probiotics have been shown to provide a protective effect, resulting in improved gut barrier function, lower stress hormone concentrations and fewer anxiety-like behaviours (Sudo et al., 2004; Brzozowski et al., 2006). Lacidofil® is a probiotic supplement containing the lactic acid bacteria *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticu*s R0052 in a 95:5 ratio (Foster, Tompkins, & Dahl, 2011) that has been demonstrated to be an effective treatment for a rat model of gastric ulceration, where the probiotic treatment reduced stress-induced responses such as increased gut permeability, increased inflammatory markers (IL-1β and TNF-α), and increased serum corticosterone levels (Brzozowski et al., 2006).

1.4 PROBIOTICS AND MATERNAL CARE

As previously discussed, maternal care behaviours are subject to alteration and adaptation to the surrounding environment and internal cues. In turn, these cues are able to differentially program the brain of offspring. The gut-brain axis and its ability to modulate hormones provides an exciting avenue for research into how microbes may affect the mother's expression of maternal care (Sarkar et al., 2016; Cowan, Callaghan & Richardson, 2016). Due to the hormonal nature of the onset of maternal care, and how subject hormones are to exterior substances, we must consider that the gut-brain axis and its microbes may play a role in the emergence of maternal care, and its regulation (Palanza, Howdeshell, Parmigiani & vom Saal, 2002; Palanza, 2017).

Maternal care is primed in the later stages of pregnancy by heightened estrogen levels (Rosenblatt, 1994). Maternal care is also associated with an increase in mesolimbic dopamine activity, which further activates estrogen receptors (Stern, 1997). The rise in estrogen precipitates an increase in oxytocin receptor binding in brain regions including the lateral septum, bed nucleus of the stria terminalis, the central nucleus of the amygdala, and the medial preoptic area of the hypothalamus (mPOA; Champagne et al., 2001). Oxytocin is a neuropeptide hormone, which is essential in self-grooming behaviour in rats, but also is a requirement for the onset of LG behaviour (Poutahidis et al., 2013; Champagne et al., 2001). This has been demonstrated by Champagne and colleagues (2001) where the infusion of an oxytocin receptor antagonist decreases LG behaviour in females.

Conversely, infusing either estrogen or oxytocin directly to the mPOA in females induces maternal care (Numan, Rosenblatt, & Komisaruk, 1977; Pedersen et al., 1994) and higher levels of oxytocin receptor binding in the mPOA are observed in dams that engage in high levels of LG (Champagne et al., 2003b). Again, this increase in oxytocin receptor binding is estrogen-dependent (Champagne et al., 2003b) and the expression of elevated estrogen receptor alpha (ER-α) is associated with high LG dams (Champagne et al., 2003b). In fact, it has been suggested that the differences observed in ER-α expression in the mPOA are perhaps a biological mechanism for the changes in oxytocin receptor binding, and ultimately, in maternal LG (Champagne et al., 2003b).

Thus, estrogen and oxytocin are vital modulators for the onset of maternal care behaviour, and, as such, maternal care can be induced by the direct infusion of estrogen or oxytocin into the mPOA of female rats (Numan, Rosenblatt, & Komisaruk, 1977; Pedersen et al., 1994). Interestingly, the gut microbiome has been shown to impact estrogen levels, due to microbes present that secrete β-glucuronidase, an enzyme that deconjugates estrogen, thereby facilitating its binding to cognate estrogen receptors that act as transcriptional regulators to implement downstream effects on the host (Plottel & Blaser, 2011). This includes estrogen binding to ER-α, which is a critical step in the initiation of maternal care (Plottel & Blaser, 2011; Champagne et al., 2003b). Specifically, lactic acid bacteria in the gut secrete β-glucuronidase, so it is possible that the ingestion of these lactic acid bacteria, such as *Lactobacillus,* may have implications for the availability of estrogen, and its ability to bind to ER-α and increase maternal care expression (Ljungh & Wadstrom, 2006).

Interestingly, there is also indirect support for the hypothesis that lactic acid bacteria ingestion results in increased hypothalamic oxytocin levels (Poutahidis et al., 2013). Poutahidis and colleagues (2013) found that ingestion of *Lactobacillus reuteri* ATC-PTA-6475 bacteria increased oxytocin levels in mice, which subsequently resulted in increased grooming behaviours. To test whether or not the observed effects were due to the introduced probiotic bacteria, some mice underwent vagotomies, thereby severing the gut-brain connection, and received probiotic treatment post-vagotomy (Poutahidis et al., 2013). It was found that vagotomised mice no longer had increased levels of oxytocin,

despite continuing to receive probiotic treatment with *Lactobacillus reuteri* ATC-PTA-6475 (Poutahidis et al., 2013).

1.5 Probiotics and the stress response

Probiotics are of interest in stress resiliency and developmental research, as it has been found that the commensal microbiota of mice can affect the development of the HPA axis, and, thus, the adult stress response (Sudo et al., 2004). Thus, colonization of the gut could be an essential factor in the prevention of later disease and dysfunction, including the malfunction of the stress response. A previous study by Ait-Belgnaoui and colleagues (Ait-Belgnaoui et al., 2012) found that the probiotic *Lactobacillus farciminus* reduced ACTH and CORT release in rats exposed to an acute stressor. In humans, there have been similar associations with stress hormones and *Lactobacillus* strains, as it was found that participants with increased cortisol levels and high reported levels of stress had an increased abundance of Proteobacteria, and lower abundance of lactic acid bacteria, such as *Lactobacillus* (Zijlmans et al., 2015).

Similarly, germ-free (GF) mice, which have no gut bacteria, show an increased HPA axis response to restraint stress, compared to specific pathogen free (SPF) and gnotobiotic mice (i.e., mice with a select microbiota introduced by the experimenter; Sudo et al., 2004). The observed increases in the HPA axis response of GF mice were corrected by reconstituting the GF mice with SPF mice feces at 3 weeks of age, an effect which persisted into adulthood, but only if the reconstitution was done prior to the mice reaching six weeks of age – later, the treatment was ineffective (Sudo et al., 2004). This suggests that there is a critical window for the early programming of the microbiome, and

its effects on the HPA axis response (Sudo et al., 2004). In support of this, the administration of probiotics during both pregnancy and lactation has shown more significant benefits over administration during lactation alone in humans (Rautava, Luoto, Salminen, & Isolauri, 2012).

In addition to the effects of probiotics on molecular outcomes such as stress hormones, previous studies have also demonstrated that offspring can have more positive behavioural results related to stress exposure (e.g., less anxiety-like behaviours) when mothers are provided probiotics during lactation (Cowan, Callaghan, & Richardson, 2016; Callaghan, Cowan, & Richardson, 2016).

There are a number of ways to model anxiety-like behaviours in animals, which can be broadly classified as either conditioned, or unconditioned tests (Rodgers, Cao, Dalvi & Holmes, 1997). Conditioned response models often require food or water restriction, and have a substantial time cost due to the training of animals to perform these tasks (Rodgers et al., 1997). Conversely, unconditioned response models, which rely on the natural tendencies of the animal (e.g., fear of open spaces), may result in individual animals' behaviours varying somewhat at baseline, prove to be more ecologically valid (Rodgers et al., 1997).

The principle of the open field test (OFT), developed by Hall (1934), is that for many mammals that are prey in the wild, rats included, a novel open environment is mildly stressful, and elicits behavioural responses such as freezing, thigmotaxis and rearing

(Denenburg, 1969; Valle, 1970). This traditional test is typically between 2-10 minutes in duration, in order to emphasize the animal's response to novelty (Gould, Dao & Kovacsics, 2009). Activity in the first 5 minutes of the test may be of particular interest when measuring anxiety, as the large arena has many features that distinguish it from the animal's ordinary environment (i.e., its dimensions, location of the testing room and the apparatus material), and the animal is also separated from its cage mates (Gould, Dao & Kovacsics, 2009).

Other tests of rat anxiety-like behaviour are the elevated plus maze (EPM), originally devised by Montgomery (1955) to determine if the novelty of an elevated maze, with open maze arms, and closed maze arms, would result in both fear and exploratory behaviours. Montgomery (1955) as well as Handley and Mithani (1984) demonstrated that in this elevated maze, rats had a strong preference for the closed arms of the maze. The administration of anxiolytic drugs, such as diazepam, was shown to significantly increase the proportion of open arm entries (Handley & Mithani, 1984).

The forced-swim test (FST) first became popularized by Porsolt and colleagues (1978) to measure depressive-like symptoms, as a means to screen pharmaceuticals to treat depression in pre-clinical animal models (de Kloet & Molendijk, 2016). The premise of the test was based on the observation that rats, when placed in a laboratory beaker full of water from which they could not escape, would often stop swimming, and float with their head just above the waterline (Porsolt, Blavet & Jalfre, 1978). At the time, it was thought that this behavioural despair was similar to human depression, however, in recent years,

the FST has since been considered as more demonstrative of coping behaviour under stress (Commons, Cholanians, Babb & Ehlinger, 2017; de Kloet & Molendijk, 2016). The sucrose preference test has been validated as a measure of the decrease in sensitivity to reward in rats in response to stress, which is considered to be analogous to the loss of pleasure suffered by people diagnosed with depressive disorders (Willner, Towell, Sampson, Sophokleous & Muscat, 1987; Willner, 1997).

In the present study, we chose to use the OFT to measure anxiety-like behaviours in adolescent offspring. Due to our interest in anxiety-like behaviours, we chose this test over the FST and sucrose preference test, as these tests are aimed at measuring depressive-like behaviours, not anxiety. Although both the OFT and EPM have been used to study anxiety-like behaviours in adolescent rats (Lynn & Brown, 2010), we chose the OFT, as we were able to look at similar behaviours between this test, and our acute stress exposure to predator odour (e.g., freezing, rearing, centre time in the OFT and vicinity to the stressor in the POE). Additionally, when considering the possibility of cross-contamination between adolescents from different maternal probiotic treatment groups, the OFT is superior, for it has considerably fewer crevices and tight spaces that may pose difficulty when disinfecting.

In a review of the literature, it was found that probiotic administration was primarily associated with a reduction in anxiety in rodent models (Wallace & Milev, 2017). For example, in a chronic stress model, rats provided *Lactobacillus helveticus* N28 stayed in the open arms of the elevated plus maze (i.e., indicative of an anxiolytic response) longer

than their control counterparts (Liang et al., 2015) and this administration was also associated with lower ACTH and CORT levels (Liang et al., 2015). In another study, adult animals that were given probiotics had lower levels of CORT 30 minutes after a 6-minute FST, as compared to their placebo-treated counterparts (Bravo et al., 2011). Heijtz and colleagues (2011) also found that in GF mice, more risky behaviour was displayed in the EPM, including more times visiting the ends of the open arms. It is postulated that the effects of probiotics on stress and anxiety may be due to probiotics' effects on improving gut epithelium integrity, which in turn limits the release of endotoxins into the blood (Wallace & Milev, 2017). Lower amounts of endotoxins mean less inflammation, which in turn leads to better HPA axis regulation (Wallace & Milev, 2017).

In addition to the abnormal behaviour observed in GF mice, these mice also have altered expression of proteins in the brain, such as synaptophysin and PSD-95, compared to SPF and colonized GF mice (Heijtz et al., 2011). Synaptophysin and PSD-95 are both involved in synaptic transmission of neurotransmitters, essential for neural communication (Wiedenmann et al., 1986; Ehrlich, Klein, Rumpel & Malinow, 2007). Not only do GF mice show alterations in neurotransmission, but adult GF mice have enlarged brain structures, including hippocampus and amygdala (Luczynski et al., 2016). Thus, it has been hypothesized that the differences in behaviour observed in GF mice are due to these observable morphological differences in brain structure, and their influence neural output to other brain regions as well (Luczynski et al., 2016). At the ultrastructural level, the amygdalae of GF mice have more axospinous synapses on the basolateral amygdala (BLA) pyramidal neurons, and it has also been observed that hippocampal

neurons including pyramidal neurons and dentate gyrus granule cells have signs of atrophy (Luczynski et al., 2016). Conversely, Ogbonnaya and colleagues (2015) have observed that GF mice have increased dorsal hippocampus neurogenesis. However, postweaning colonization of GF mice does not prevent this increase in neurogenesis, which suggests that there is a critical period where the microbiota influence the brain (Ogbonnaya et al., 2015).

Further interrogation of how HPA axis programming occurs in early life and the impact that probiotics may have has been performed using early life stress (ELS) models in rodents. In rodents, ELS generally leads to increases in anxiety- and depression-like behaviours in adult animals and can be induced in different ways (Cui et al., 2006; Dalle Molle et al., 2012; Lippmann et al., 2007). However, it often involves removing the offspring from the mother for varying periods throughout early life (Levine, 1957; Lippmann et al., 2007; Murthy & Gould, 2018).

Using an ELS model in mice, it was found by Liu and colleagues (2016) that *Lactobacillus plantarum* PS128 given in adulthood had positive effects on ELS-exposed mice. Before probiotic treatment, ELS mice had anxiety-like and depression-like behaviours, which were demonstrated in the OFT, EPM, FST and sucrose preference tests (Liu et al., 2016). Consistent with their behaviour, ELS mice had elevated serum CORT levels, compared to their experimentally naïve counterparts (Liu et al., 2016). After a 4-week treatment with *L. plantarum* PS128, ELS mice had reduced depression-like behaviour and a normalized stress response (Liu et al., 2016) However, probiotic

treatment did not decrease anxiety-like behaviour in ELS mice, but it did reduce anxiety-like behaviour in the ELS-naïve controls, who also had probiotic treatment (Liu et al., 2016).

Taking into consideration what we know from GF, ELS and human studies, in combination with probiotic treatment, Lactobacilli have implications in the programming of a "healthy" stress response and the gut microbiome. Furthermore, the benefits of a healthy microbiome appear to have a critical window in early life, where they make an impact on later outcomes, such as stress responding and anxiety-like behaviours. If these benefits were able to be passed on by the mother, through the known transfer of commensal bacteria at birth, there could be profound implications for offspring stress resiliency (Asnicar et al., 2017).

As discussed, previous maternal care research has demonstrated profound effects of diet, hormones, and postnatal environment on the behaviours provided by the mother to her offspring (Champagne et al., 2003a; Francis & Meaney, 1999; Korgan et al., 2016; Korgan et al., 2018; Connor et al., 2012).

To date, there has been no investigation as to whether or not probiotics result in positive effects on maternal care behaviours in rats (e.g., increased arched-back nursing and licking/grooming), subsequently affecting offspring stress and anxiety-like outcomes. The first objective of this thesis was to investigate if probiotic treatment of dams throughout pregnancy and lactation resulted in higher frequencies of active maternal care

(LG/ABN). The second objective of the thesis was to examine if probiotic treatment received in early life through the dam had positive effects on stress resiliency for offspring, including reduced anxiety-like behaviours, and lower stress hormone levels and lower inflammation levels in response to an acute stressor (i.e. lower ACTH, CORT, and inflammatory markers).

CHAPTER 2 MATERIALS AND METHODS

2.1 ANIMALS AND BREEDING

For the purposes of this experiment, 35 Long-Evans hooded rats, 17 males and 18 females (~60 days old) were purchased from Charles River Laboratories (St. Constant, Quebec). Upon their arrival, animals were given 9-12 days to acclimate to the facility prior to the commencement of the experiment (see Figure 1 for the experimental timeline). Initially, rats 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). Food (Laboratory Rodent Diet 5001, LabDiet®, St. Louis, MO, United States of America) and tap water were provided *ad libitum.* Prior to the commencement of this experiment, water quality was tested for common analytes that may affect probiotic treatment. The results of water testing were normal.

Animals were initially housed in a single colony room for the aforementioned acclimation period, with a 12h:12h reverse light-dark cycle (lights off at 1000h). The temperature of the colony room was maintained at 21ºC ± 2ºC.

Animals were then randomly assigned to one of three experimental conditions: placebo, probiotic, or naïve control, and moved into a colony room designated for their experimental condition, to reduce the risk of cross-contamination between groups. The majority of the animals were assigned to either the placebo (n = 15), or the probiotic condition (n = 16). The naïve control group consisted of only 4 animals (2 males, 2

females) and was used to generate a few samples specifically required for a later real-time quantitative polymerase chain reaction (qPCR) experiment.

Following animals moving into their designated colony room, male and female rats were paired for breeding purposes by placing one male with a single female for seven consecutive days. All colony rooms had the same 12h:12h reverse light-dark cycle (lights off at 1000h), and temperature maintained at 21ºC ± 2ºC. After the seven-day breeding protocol, females were once again housed in pairs until estimated gestational day 15 (GD15), at which time they were singly housed in preparation for giving birth. Beginning at GD18, the arrival of litters was monitored twice daily, once at 0900h, and once again at 1500h. Once a litter was born, this day was designated as postnatal day 0 (PD0), and the litter was sexed and weighed.

All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) and were approved by the Dalhousie University Committee on Laboratory Animals (UCLA) under animal ethics protocol #18-022.

2.2 Maternal Probiotic Administration

This experiment included daily probiotic (or placebo) administration to dams during pregnancy and lactation, for a total of 42 days.

Prior to the commencement of daily probiotic administration, dams had to be trained to orally receive probiotic or placebo solution via syringe as per Tillman & Wegner, 2018.

This included four days of training, such that prior to breeding, females were trained to feed from a 1 millilitre (mL) oral syringe using a 0.25M sucrose solution. Each female rat was administered 0.5 mL of 0.25M sucrose solution for four days.

The day that breeding pairs were separated was considered gestational day zero (GD0). On GD0, placebo and probiotic administration began, and continued through gestation (~21 days), until offspring were weaned from their dam (21 days) for a total of 42 treatment days. The probiotic solution was prepared at a dosage of one billion (10^9) colony-forming units (CFU) per 0.5 mL of reverse-osmosis water daily. Each rat was given 0.5 mL of solution (0.0192 grams of powder).

$$Daily\ dose\ per\ rat = \frac{0.5\ mL\ dH_2O}{1} \times \frac{10^9\ CFU}{1\ mL\ dH_2O} \times \frac{x\ grams\ Lacidofil®}{2.6^{10}\ CFU}$$

$$Daily\ dose\ per\ rat = 0.0192\ grams\ Lacidofil®$$

2.3 Litter Sizes

At postnatal day 21, offspring were weaned from their dam. For the purposes of behavioural testing, two male and two female offspring were kept for this experiment. Remaining offspring were utilized in other experiments or sacrificed to use tissue for optimization purposes. The final sample sizes for the two experimental groups of offspring in this experiment were 28 placebo-treated offspring and 31 probiotic-treated offspring. The breakdown of female and male offspring for each treatment was the following: n = 14 placebo-treated females, n = 14 placebo-treated males, n = 16 probiotic-treated females and n = 16 probiotic-treated males. One male from the

probiotic-treated group died unexpectedly, after the open field test, but before the end of the experiment, resulting in $n = 15$ probiotic-treated males.

2.4 Maternal Care Observations

All dams and their litters were observed for their maternal care behaviours, starting on the day after birth, postnatal day 1 (PD1), for seven consecutive days (until PD7). Daily observations were scored manually in real-time by a trained observer for 60 minutes at five timepoints throughout the day: 0800h, 1100h, 1300h, 1500h, and 2200h. For all observation periods, the frequency of maternal care behaviours was recorded every three minutes (Korgan et al., 2016). 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 A for the ethogram used for behavioural scoring).

2.5 Anxiety-Like Behaviour Testing of Juvenile Offspring

All offspring ($n = 60$) that were kept postweaning were tested for anxiety-like behaviours on post-natal day (PD) 32-35.

To observe anxiety-like behaviour, the open field test (OFT) was used to assess the following behaviours: frequency of line crosses, frequency of rearing, duration of thigmotaxis, frequency of freezing, duration of center time and latency to move from the center. The OFT consisted of a square arena, constructed from black Plexiglas® (72.9 x 78.9 x 35.0 cm; see Figure 2 for a diagram of the apparatus). Sixteen equal-sized quadrants, measuring 19.7 cm x 19.8 cm, were designated on the maze floor by white

Fisherbrand® tape. All trials were performed during the dark phase of the light cycle, beginning no earlier than 1000h, and ending no later than 1800h. Each trial was 10 minutes in duration. The OFT arena was illuminated by two red lights, and all trials were recorded with a video camera (Sony Handycam®, DCR-SR68), mounted vertically above the arena on a tripod, for future behavioural scoring (see Appendix B for the scoring sheet). All behaviours were scored manually by a trained observer, from videos of the trials. Each behaviour was operationally defined in an ethogram prior to the start of scoring (see Appendix C for the ethogram). Rats were transported in a covered transport cage to a separate behavioural testing room containing the OFT arena. Twenty-four hours prior to the commencement of testing, the behavioural testing room was cleaned using Prevail® and 70% ethanol, to lessen the risk of cross-contamination between the placebo and probiotic animals. To further ensure that cross-contamination did not occur, placebo animals were tested prior to probiotic animals. Before the first trial of each testing day, the arena was cleaned using 70% ethanol, to ensure all animals were tested under similar conditions.

2.6 Acute Stress Exposure of Peri-Adolescent Offspring

Between 8 and 11 days after behavioural testing in the OFT, on PD 40-43, the same offspring (n = 59) were exposed to an acute stressor. The stressor consisted of a ~1 cm long piece of nylon cat collar (Great Choice, Breakaway Cat Collar), which had been worn by a reproductively intact female cat for one week prior to the behavioural test. Upon collection of the cat collars from the female cats, the collars were cut into ~1 cm-long pieces, placed in double resealable plastic bags, and stored at -30^{o}C until use (Perrot-Sinal et al., 2004).

All trials were 30 minutes long and took place in a designated testing room, under red light, between the hours of 1000h and 1800h. Before each trial began, 70% ethanol was used to wipe down the testing arena, to remove olfactory cues and prevent cross-contamination between groups. Once the arena was clean, a fresh ~1 cm piece of cat collar was clipped to one side of the interior of a Plexiglas® testing arena (60 cm x 27 cm x 35.5 cm) with a white plastic floor and a clear Plexiglas® lid (with ventilation holes; see Figure 3 for a diagram of the apparatus). For each trial, offspring were transferred to the testing room in a covered transport cage and placed in the clean test arena. All trials were recorded using a video camera mounted on a tripod level with the testing arena for future behavioural scoring. All behaviours were scored manually by a trained observer from these videos (see Appendix D for the scoring sheet). The behaviours scored included: frequency of supported rearing, frequency of unsupported rearing, duration of rearing, duration in stimulus vicinity, frequency of stimulus contacts, frequency of grooming and frequency of freezing (see Appendix E for the ethogram). After each trial, rats were transported to a quiet holding room prior to sacrifice and tissue collection.

2.7 Sacrifice and Tissue Collection

Dams were sacrificed five days after their offspring were weaned, on PD 26. Until the dams were sacrificed, they continued to receive either placebo, or probiotic treatment as assigned. Dams were sacrificed within 0-8 hours of receiving treatment. Dams were first deeply anesthetized with Euthanyl® (sodium pentobarbital, 60 mg/kg, intraperitoneal). Animals were then quickly decapitated, trunk blood was collected, followed by brains and internal organs. Brains were flash frozen on dry ice, and coronal gross dissections were taken of the hypothalamus and hippocampus, using the rat brain atlas from Paxinos

and Watson as a guide (Plates 27-37; Paxinos & Watson, 1998). Brain samples were stored at -80ºC until further processing. Internal organs including the stomach, small intestine and large intestine (including both caecum and colon) were removed quickly and flash frozen on dry ice. Plasma samples were centrifuged immediately post-collection. An initial centrifugation was performed at 1,000 x *g* for 15 minutes at 4ºC and the supernatant was transferred to a new 1.5 mL microtube. A second centrifugation step was performed at 10,000 x *g* for 10 minutes at 4ºC. Upon completion of the second centrifugation, the plasma samples were transferred to a new 1.5 mL microtube and were then stored at –80ºC until use.

All internal organs were stored at -80ºC until further processing. Offspring were sacrificed on PD 40-43, at 1-hour post-stress exposure using the same procedure used to collect samples from dams.

2.8 Sample Preparation For Multiplex Analysis

All hypothalamus samples were weighed prior to proceeding with preparation for protein extraction. The hypothalamus samples taken from offspring from placebo-treated dams weighed an average of 60.54 micrograms (µg) with a standard deviation of 39.37 µg. The hypothalamus samples taken from offspring from probiotic-treated dams weighed an average of 57.74 µg, with a standard deviation of 18.17 µg. Using forceps, each hypothalamus sample was placed into a 2 mL bead beating tube (Sarstedt, 72.693) containing four to five 3 mm glass beads (Fisher, 11-312A).

Lysing solution was prepared by adding 1 mL Cell Lysis Buffer (from Bio-Rad Kit #171-304012) with Protease Inhibitor and 4 µL of 500 mM PMSF (Sigma P7626-250mg) dissolved in dimethyl sulphoxide (Sigma D2650-100mL) to each bead beating tube. The samples were then homogenized using a MP FastPrep-24^{TM} 5G bead homogenizer. A pre-programmed protocol for mouse brain was used, with one 40-second cycle of 6.0 m/s.

After the samples were homogenized, they were centrifuged at 11,000 RPM for 10 minutes at 4ºC. The supernatant was pipetted into a new microtube and the samples were then stored at -20ºC until further analysis.

Total protein concentration (µg) was determined using a Bradford Protein Assay. Briefly, a standard curve (5 µg/µL, 4 µg/µL, 3 µg/µL, 2 µg/µL, 1 µg/µL, 0.5 µg/µL and 0.2 µg/µL, and 0 µg/µL) was generated using a stock solution of bovine serum albumin (BSA), and including lysis buffer and reverse osmosis water. Hypothalamus samples were diluted to a 1:50 dilution with reverse osmosis water. The standard curve was assayed in triplicate, and samples were assayed in duplicate, at an absorbance of 595 nm after being exposed to Bradford reagent (Sigma B6916-500mL). ThermoFisher Multiskan Ascent® software (ThermoFisher, USA) automatically calculated total protein concentration for each sample.

2.9 Multiplex Analysis of Stress Hormones

The Luminex® bead-based assay, MilliPlex MAP Rat Stress Hormone Magnetic Bead Panel (Cat# RSHMAG-69K; MilliPoreSigma), used to detect stress hormones ACTH, CORT and melatonin, in both hypothalamus and plasma samples from offspring. The

assays were prepared according to manufacturer's instructions. Briefly reagents, standards and quality controls were reconstituted using the appropriate diluents and incubated for 10 minutes at room temperature. Standards, controls and blanks (assay buffer) were added to the provided 96-well plate. Assay buffer was then added to the sample wells, along with the appropriate sample matrix, followed by the samples themselves. For the brain tissue samples, the sample matrix used was the sample diluent from the Bio-Rad kit used for the protein extraction protocol. For the plasma samples, the sample matrix used was the serum matrix provided with the MilliPlex® assay kit. The antibody-immobilized beads were then vortexed and added to all wells. The plate was sealed and incubated overnight (18 hours) on a plate shaker, protected from light, set at 500 RPM, at 4ºC.

On the second day of the assay, the plate was removed from the incubator, and placed on a handheld magnet to ensure the beads stayed in the plate wells. The plate was then washed three times using wash buffer. Detection antibodies were added to each well, the plate was sealed, and then incubated with agitation on the plate shaker for 1 hour, at 500 RPM, at room temperature. After this incubation, the detection antibodies were left in the wells, and Streptavidin-Phycoerythrin was added to each well. The plate was again sealed and incubated with agitation for 30 minutes, 500 RPM, at room temperature. Post-incubation, the plate was placed on the handheld magnet, the plate was washed three times, and sheath fluid from the BioPlex® 200 system (Bio-Rad) was added to each well of the plate. The plate was then sealed, the beads were resuspended by shaking on the plate shaker at 500 RPM for 5 minutes. Lastly, the plate was run on the BioPlex® system using the program settings provided by MilliPoreSigma.

2.10 Multiplex Analysis of Inflammation Markers

The Luminex® bead-based assay, Bio-Plex Pro™ Rat Cytokine 23-Plex Assay (Cat#12005641; Bio-Rad), was used to detect 23 inflammation markers found in plasma samples from both dams and their offspring (see Tables 1 and 2 for a list of inflammation markers and their functions; adapted from the Rat Genome Database; Shimoyama et al., 2014). The assay was prepared according to manufacturer's instructions. 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 prior to preparing the standard curve in a dilution series. The antibody-coupled magnetic beads were vortexed, diluted using assay diluent, 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 magnet, 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 to place the plate 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 washed three times again, and 50 µL Streptavidin-Phycoerythrin was added to each well. The plate was once again incubated at 850 RPM at room temperature, for 10 minutes. The plate was washed three times, and then the magnetic beads were resuspended in assay

diluent, put on the plate shaker at 850 RPM for 30 seconds, and then read in the BioPlex® 200 system.

2.11 Real-Time Quantitative PCR (qPCR) Analysis

Real-time quantitative PCR (qPCR) was used to test caecum samples from dams and their offspring to determine whether the probiotic strains *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 were present in the caecum samples of the probiotic-treated mothers and their offspring, and if there had been any cross-contamination between placebo and probiotic animals.

2.11.1 DNA Extraction

Genomic DNA (gDNA) was isolated from caecum samples of dams and their offspring using a DNA extraction kit: the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany). Samples were processed according to a standard operating procedure and the manufacturer's instructions (MacPherson et al., 2018). All reagents, with the exception of the phosphate buffered saline (PBS), were included in the DNA extraction kit. First, 150-400 mg of caecum sample from dams and offspring were weighed out and placed into individual bead beating tubes, containing four to five glass beads. Next, 500 µL of PBS was added to each tube, and the samples were vortexed to homogenize the sample. Another 500 µL of PBS was then added, and the sample homogenized via vortexing again. Samples were then centrifuged for 1 minute at 13,000 RPM. Following centrifugation, the supernatant was discarded, and the pellet was washed using 1 mL of PBS. Another centrifugation step for 1 minute at 13,000 RPM was then completed, and the supernatant discarded again. Each sample then received 1 mL of InhibitEX and was vortexed and set to incubate at 70°C in a dry bath for 5 minutes.

After the incubation step, ~300 mg of zirconia beads was added to each sample tube. The samples were then homogenized using a bead homogenization protocol (using a MP FastPrep-24^{TM} 5G bead homogenizer) with three rounds of 60-second homogenization at 4 m/s, with a 60-second rest period in between each round. After the bead homogenization step, the samples were centrifuged for 3 minutes at 13,000 RPM to pellet the particles. Then, 600 µL of the supernatant was transferred into a labelled microcentrifuge tube and centrifuged again for 3 minutes at 13,000 RPM to pellet any inhibitors from the prior InhibitEX treatment. Next, 25 µL Proteinase K was added to a new microcentrifuge tube, and 400 µL of the sample's supernatant was added to the new tube containing Proteinase K. Once the sample was transferred, 400 µL of Buffer AL was added and the sample was vortexed for 15 seconds. All samples were then incubated in a dry bath at 70^{o}C for 10 minutes. Next, 400 µL of 100% ethanol was added to the lysate, and vortexed. The lysate was then transferred in two rounds of loading 600 µL of lysate onto a QIAamp spin column and centrifuged at 14,800 RPM for 1 minute. The flow-through from the spin column was discarded after centrifugation, and this process was repeated again to transfer all the lysate to the spin column. Then, 500 µL of Buffer AW1 was added to the spin column and the samples were spun at 14,800 RPM for 1 minute. Following this step, 500 µL of Buffer AW2 was added to the spin column and centrifuged at 14,800 RPM for 1 minute. The spin column was then transferred onto a new 2 mL microcentrifuge tube, and 50 µL of Buffer ATE was pipetting directly onto the spin column's membrane. All samples were then incubated for 5 minutes at room temperature, and then centrifuged at 14,800 RPM for 1 minute to elute the DNA. After the extraction process, the DNA concentration (ng/µL) and purity (260/280 ratio) of each

sample was assessed using spectrophotometry. All DNA samples were stored at -20ºC until further analysis.

2.11.2 Spiking Experiment

A spiking experiment was performed using naïve control caecum samples, which were from animals that had not received placebo or probiotic and had been housed separately. The spiking experiment was used to generate a standard curve with known concentrations of *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 bacteria, in order to quantify unknown samples in a separate qPCR experiment.

First, a spiking solution was prepared, containing approximately one gram of lyophilized powder of the *Lactobacillus rhamnosus* R0011 or *Lactobacillus helveticus* R0052 strain and 99 mL of phosphate buffered saline (PBS). This solution was then serially diluted, and flow cytometry was performed to obtain cell counts of dilutions #5 and #6 (BD Accuri cytometer, with SYTO® 24 fluorescent nucleic acid labelling). The volume of bacterial suspension was calculated using the formula:

$$Total\ Count = Flow\ Count\ \times\ Dilution$$

$$\frac{Total\ count\ \times\ 10^{-2}}{1\ mL} = \frac{10^9}{x}$$

$$x\ = Volume\ of\ 10^{-2}\ dilution\ required\ to\ obtain\ 10^9\ target\ bacteria$$

The naïve control caecum samples were then weighed out to one gram per sample and combined together to form one homogenous spiking matrix. Approximately 250 mg of the matrix was then weighed out into four different 2 mL bead beating tubes, containing 4

to 5 sterile 3 mm glass beads. The volume of bacterial suspension calculated in the above equation was then pipetted into three of the four 2 mL tubes to make spiked samples. One of the tubes was maintained as a naïve control sample and did not have bacterial suspension added to it. The same DNA extraction protocol as described previously was then implemented.

2.11.3 Strain Detection Experiment

The DNA extracted from the spiking experiment was diluted from 10^9 bacteria, in a 10-fold dilution, down to 10^4 bacteria, to generate a standard curve. The master mix was prepared using SYBR green (Select Master Mix, Life Technologies, 4472980). The unknown samples were diluted to a 1:5 dilution, with molecular biology grade water, to serve as the DNA template for qPCR. All pipetting, including master mix and template DNA was performed by an automated pipetting/ liquid handling system (ep*Motion*® 5075t, Eppendorf). Standards were run in duplicate, and samples were run in triplicate on a 384-well plate. Cycling conditions were as follows: 50°C for 2 minutes, 95°C for 2 minutes, 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds (40 cycles).

2.12 Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistics (Version 25, New York, USA). Data visualization was performed in R (Version 3.5.1), using the following packages: ggplot2, ggthemes, ggExtra, extrafont, foreign, ggsignif and gridExtra (Wickham, 2016; Arnold, 2018; Attali & Baker, 2018; Chang, 2014; R Core Team, 2018; Ahlmann-Eltze, 2019; Auguie, 2017).

The effects of probiotic treatment on litter birth weight, male ratio and total number of offspring per litter was analyzed using Mann-Whitney U tests. The effects of probiotic treatment on offspring wean weights was analyzed using a two-way analysis of variance (ANOVA) with offspring sex, and maternal treatment as independent variables. The significance criteria was set *a priori* at $p < 0.05$.

The effects of probiotic treatment on maternal care behaviours were analyzed using Mann-Whitney U tests, using the Benjamini-Hochberg correction for multiple comparisons (Benjamini & Hochberg, 1995).

The effects of maternal treatment on offspring anxiety-like behaviour was analyzed using a two-way ANOVA with offspring sex, and maternal treatment as independent variables. The effects of maternal treatment on offspring stress responding behaviours was analyzed using a two-way ANOVA, with offspring Sex and Maternal treatment as independent variables. Multiple comparisons were corrected for using a Bonferroni correction.

To determine if the independent variables sex and maternal treatment had effects on the three stress hormones measured in offspring plasma (ACTH, CORT and melatonin), we performed a two-way ANOVA on each analyte, with a Bonferroni correction to correct for multiple comparisons. If there was an interaction between sex and treatment, a simple effects analysis was performed to probe the interaction.

To determine if the independent variables sex and maternal treatment had effects on the 23 inflammation markers analyzed in offspring plasma samples, we performed a two-way ANOVA on each analyte. We performed Bonferroni corrections for our planned multiple comparisons. Interactions between sex and treatment were probed using simple effects analysis.

To compare offspring plasma sample stress hormone levels and inflammation markers, Spearman's correlations were used. Active maternal care behaviours, ABN and LG, were also compared to inflammation markers using Spearman's correlations. For all correlations, significance level was set *a priori* at $p < 0.01$.

CHAPTER 3 RESULTS

3.1 Probiotic Treatment Effects on Offspring Weights

We found no effects of maternal probiotic administration on litter size, litter sex ratio or mean birth weights (see Table 3). We did find a significant main effect of treatment on weaning weight ($F(1, 56) = 7.65$, $p = .008$, $\eta_p^2 = .120$), where offspring from probiotic-treated dams weighed more ($M = 51.89$; $SD = 7.92$) than offspring from placebo-treated dams ($M = 45.76$; $SD = 9.10$).

3.2 Probiotic Treatment Effects on Maternal Care

We did observe that dams in the probiotic treatment group behaved differently towards their offspring compared to placebo group dams, as demonstrated by differences in the frequencies of maternal behaviours observed. Due to the non-normally distributed nature of the data, and the small sample size (less than 30 animals), Mann-Whitney U tests were performed. Using the Benjamini-Hochberg correction, significance was set at $p < 0.005$. Any p-values less than .05 were considered trending effects.

Probiotic-treated dams engaged in higher frequencies of ABN ($Mdn = 229.00$), as compared to their placebo-treated counterparts ($Mdn = 157.00$), $U = 52.00$, $p = 0.004$, $r = .72$ (see Figure 4A). Probiotic-treated dams also trended towards higher frequencies of LG ($Mdn = 83.50$), when compared to their placebo-treated counterparts ($Mdn = 57.00$), $U = 82.50$, $p = 0.029$, $r = .55$ (see Figure 4B). Probiotic-treated dams also trended towards displaying higher frequencies of self-feeding bouts ($Mdn = 121.00$), compared to placebo-treated dams ($Mdn = 106.00$) $U = 46.50$, $p = 0.029$, $r = .55$ (see Figure 4C).

Interestingly, probiotic- and placebo-treated dams did not significantly differ in the amount of time spent engaging in more passive nursing behaviours such as PN and BP (see Table 4). Dams also did not differ in the amount of passive contact spent with their litters when not nursing (see Table 4). Dams also did not differ based on their treatment in the amount of time spent with no contact from their pups (NCP), or in their time engaging in other care behaviours such as NB and SG (see Table 4). Lastly, dams did not differ based on treatment in the frequency of separation/retrieval behaviours, SP and PR, respectively (see Table 4).

3.3 Maternal Treatment and Offspring Anxiety-Like Behaviour

Offspring anxiety-like behaviours were tested in the OFT, and all behaviours except for frequency of freezing across the 10-minute test were analyzed using two-way ANOVA to assess the effects of maternal treatment and sex, and any interaction between these two factors. Frequency of freezing was not analyzed, as the majority of animals did not demonstrate any freezing behaviours. Due to the number of behaviours assessed, a Bonferroni correction was used, setting the corrected p-value at $p < 0.003$, and p-values less than 0.05 were considered trending effects.

Two-way ANOVA revealed no significant main effects for sex, or maternal treatment for any of the anxiety-like behaviours assessed using the OFT. In addition, no interaction effects between sex and maternal treatment were observed (see Tables 5 and 6).

3.4 Maternal Treatment and Offspring Stress-Responding

The predator odour exposure (POE) test was analyzed first as a whole (i.e., the entirety of the 30-minute exposure was analyzed), and then we examined this test further by

analyzing each 5-minute bin (see Appendix D). Due to the nature of multiple comparisons, we corrected for multiple comparisons using a Bonferroni correction to set the accepted significance level at $p < 0.002$, and p-values < 0.05 were considered trending effects.

No significant differences were found for any of the observed behaviours in the POE test, when the total time for the test was analyzed.

Interestingly, we found two trending effects in minutes 5-10, designated "bin 2" of the test. We observed a trending main effect of treatment ($F(1, 52) = 4.13$, $p = .047$, $\eta_p^2 = .074$) where offspring from probiotic-treated dams spent less time rearing ($M = 3.41$; $SD = 6.75$), when compared to their counterparts from placebo-treated dams ($M = 8.50$; $SD = 11.25$; see Figure 5A). We also found a trending main effect of treatment ($F(1, 52) = 4.54$, $p = .038$, $\eta_p^2 = .080$) such that offspring from probiotic-treated dams contacted the predator odour stimulus less ($M = .27$; $SD = .583$) than their placebo-reared counterparts ($M = .81$; $SD = 1.23$; see Figure 5B).

When the first half (minutes 0-15, designated "bins 1-3") of the POE test was analyzed we observed a trending main effect of sex on grooming frequency ($F(1, 55) = 4.86$, $p = .032$, $\eta_p^2 = .081$) where males groomed more ($M = 4.38$; $SD = 4.62$) than females ($M = 2.03$; $SD = 3.34$; see Figure 5C).

In the latter half of the POE test (minutes 16-30, designated "bins 4-6") we found a trending main effect of treatment $F(1, 53) = 6.60$, $p = .013$, $\eta_p^2 = .111$) such that probiotic-reared offspring contacted the predator odour stimulus less ($M = .34$; $SD = .897$) than their placebo-reared counterparts ($M = 1.25$; $SD = 1.67$; see Figure 5D).

3.5 Maternal Treatment Effects Offspring Stress Hormones

3.5.1 Stress Hormone Levels in Offspring Plasma Samples

In offspring plasma samples, two-way ANOVA, with a Bonferroni correction setting the accepted p-value at $p < 0.017$, revealed no significant differences in ACTH or CORT levels (see Figures 6A and 6B). Female offspring from placebo-treated dams showed a trend towards higher melatonin levels ($M = 163.75$; $SD = 179.44$, $F(1, 42) = 5.614$, $p = .022$, $\eta_p^2 = .118$), compared to their placebo-treated male offspring counterparts ($M = 58.54$; $SD = 95.07$). The descriptive statistics for all stress hormone analytes are detailed in Table 7, and the two-way ANOVA results are detailed in Table 8.

3.5.2 Stress Hormone Levels in Offspring Hypothalamus Samples

In offspring hypothalamus samples there were no significant differences in any of the analyte levels based on sex, treatment, or an interaction of sex and treatment (see Tables 7 and 8 for descriptive statistics and two-way ANOVA results, respectively).

3.6 Inflammation Marker Levels

There were no significant differences between inflammation marker levels between placebo- and probiotic-treated dams. Active maternal care behaviours were found to be negatively correlated with some inflammation markers. LG was negatively correlated with both IL-2 ($r_s = -.535$, $p = .04$) and IL-6 ($r_s = -.548$, $p = .034$; see Figures 7A and 7B,

respectively). ABN was negatively correlated with MIP-3α (r_s = -.574, p = .025; see Figure 7C).

In offspring plasma samples we found a trending interaction between sex and maternal treatment, where male offspring from probiotic-treated dams had lower levels of GRO/KC (M = 81.17; SD = 88.48, $F(1, 53)$ = 4.430, p = .049, η_p^2 = .071.), compared to their male offspring counterparts from placebo-treated dams (M = 152.82; SD = 121.79). There were many significant correlations at the $p < 0.01$ level between offspring plasma inflammation marker levels and offspring plasma stress hormone levels (see Table 9).

3.7 Probiotic Strain Detection

Strain detection of probiotic bacteria using qPCR revealed that *Lactobacillus rhamnosus* R0011 from the probiotic formulation Lacidofil® was detectable in dams given the probiotic (M = 6.74 LOG bacteria per gram of caecum sample; SD = .58) but was beyond the limit of detection in placebo-treated dams (see Figure 8A). Strain detection of probiotic bacteria using qPCR also found that *Lactobacillus helveticus* R0052 was detectable in probiotic-treated dams (M = 5.89 LOG bacteria per gram of caecum sample; SD = .47) but was beyond the limit of detection in placebo-treated dams (see Figure 8B). Lacidofil® bacteria were beyond the limit of detection in all offspring, regardless of the treatment condition of their dam.

CHAPTER 4 DISCUSSION

4.1 General Discussion

The overall goal of the present study was to examine whether altering the gut microbiota during early life, via the mother, is sufficient to observe benefits in otherwise healthy offspring. Due to the importance of maternal care for programming neuroendocrinological systems in offspring, our first objective was to investigate maternal care behaviours provided to offspring and how those behaviours were affected by exposure to probiotic treatment throughout pregnancy and lactation. The second objective was to determine if the maternal probiotic treatment had programming effects on offspring with respect to basic physiological outcomes (e.g., body weight), anxiety-like behaviours, behavioural response to an acute stressor, stress hormone release and inflammation levels. The results showed that dams treated with probiotics throughout pregnancy and lactation engaged in significantly higher frequencies of ABN, and higher trending frequencies of LG, compared to their placebo-treated counterparts. At weaning, offspring from probiotic-treated dams were heavier than offspring from placebo-treated dams. The probiotic strains used were detectable in probiotic dams only, with no detection of the strains found in either placebo-treated dams, or offspring from either condition. There were no differences in anxiety-like behaviour in offspring, and only a few trending differences between offspring stress responding behaviours. Female offspring from placebo-treated dams had higher levels of melatonin, compared to male offspring from placebo-treated dams, but there were no significant differences in the levels of ACTH or CORT found in the offspring. Interestingly, offspring from probiotic-treated dams showed many significant negative correlations between CORT and various

inflammatory markers (both pro- and anti-inflammatory markers), whereas offspring from placebo dams did not.

While there were no observable effects of maternal probiotic treatment on offspring birth weights, litter size or litter sex ratios, offspring from probiotic-treated dams were significantly heavier at weaning (PD 22) than offspring from placebo-treated dams. This finding is consistent with increased food intake found in a previous study that provided *L. rhamnosus* GR-1 and *L. fermentum* RC-14 to pregnant rat dams over 30 days (Anukam et al., 2005). Anukam and colleagues (2005) observed that dams fed probiotics had a 12-14% increase in their food intake, which was also reflected in the offspring from probiotic mothers having an increased post-weaning food intake as well. A second study by Yakabe and colleagues (2011) also found that pregnant rat dams given a 10^8 CFU dose of *L. breve* KB290 for two weeks consumed more food compared to their control counterparts, who were given the vehicle formula only. Increased weight with probiotic supplementation has also been demonstrated in a pig model, where pigs fed a multi-probiotic containing *L. plantarum* ATCC 4336, *L. fermentum* DSM 20016 and *Enterococcus faecuim* ATCC 19434 weighed significantly more than controls (Veizaj-Delia, Piu, Lekaj & Tafaj, 2010), and this finding has been demonstrated in many other pig studies to date (Dowarah, Verma & Agarwal, 2017). The work from these non-human animal models is consistent with findings from clinical trials, where *Lactobacillus* strains given to pregnant women modified children's weight gain during the first six months of life (Luoto et al., 2010). Although we did not directly measure food intake in the present study, based on our finding that offspring from probiotic-treated dams weighed

significantly more at weaning and our observation that probiotic-treated dams engaged in more self-feeding behaviours than their placebo counterparts during our maternal care observation periods, it is reasonable to hypothesize that probiotic treatment resulted in increased food intake in the present experiment as well.

One means by which probiotic supplementation to the mother can affect offspring wean weight is directly via her milk. Consuming the probiotic can improve nutrient digestion in the gastrointestinal (GI) tract of the dam as *Lactobacillus* bacteria species produce lactic acid and proteolytic enzymes, (Veizaj-Delia, Piu, Lekaj & Tafaj, 2010; Giang, Viet, Ogle & Lindberg, 2010) and it has been established that nutritional changes alter the composition of maternal milk in rats (Keen et al., 1981; Del Prado, Delgado & Villalpando, 1997; Priego, Sanchez, Garcia, Palou & Pico, 2013). For example, high-fat diets (HFD) delivered to dams throughout pregnancy and lactation have been shown to increase daily energy intake, increase daily milk volume, and increase milk lipid concentration (Del Prado, Delgado & Villalpando, 1997). Maternal nutrition also affects the relative balance of different oligosaccharides, fermentable biomolecules, found in maternal rat milk where dams fed a high prebiotic fibre diet versus a high protein diet had differential oligosaccharide profiles (Hallam et al., 2014). Similarly, in humans, it has been found that probiotic supplementation (a combination of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bb99 and *Propionibacterium freudenreichii* subspecies *shermanii* JS) increases the abundance of 3-fucosyllactose and 3'-sialyllactose, two oligosaccharides found in human breast milk (Seppo et al., 2019). Another study in humans combining rapeseed oil-based fatty acid supplements and a probiotic formulation

of *L. rhamnosus* GG and *B. lactis* Bb12, found that participants receiving both fatty acids and probiotics had higher γ-linolenic acid concentrations (Hoppu et al., 2012). These results provide an exciting avenue for future research into how probiotics may change breast milk composition, as observed with other dietary modifications, thus affecting offspring programming via maternal nutrition (Seppo et al., 2019; Anukam et al., 2005; Hoppu et al., 2012).

In the present study, we hypothesized that a probiotic treatment, Lacidofil®, administered throughout pregnancy and lactation, would increase the frequency of active maternal care behaviours provided to offspring. We found that probiotic treatment provided to dams throughout pregnancy and lactation increased the active maternal care behaviours, LG/ABN, as well as self-feeding behaviour. It is still unclear how the gut microbiota might influence neuroendocrine function and, as a result, behaviours associated with endocrine changes, such as pregnancy (Cussotto, Sandhu, Dinan & Cryan, 2018). Increasing evidence points to the ability of these microbes to produce metabolites, including hormone-like short-chain fatty acids (SCFAs), neurotransmitters and GI hormones, and to exert indirect action and modulation on inflammatory/immune responses and hormone secretion (Cussotto, Sandhu, Dinan & Cryan, 2018). Although the goal of the present study was not to elucidate mechanism, this would be an exciting avenue for future research.

Despite not knowing how probiotics change maternal behaviour, the fact that they were able to do so is consistent with the plasticity known to exist in such behaviour and has

implications for offspring outcomes. Rat maternal care has been demonstrated in many studies to be variable in laboratory rats, under stable lab conditions (McLeod, Sinal & Perrot-Sinal, 2007). However, maternal care behaviours are also plastic, and are altered in response to many different external, or internal, stimuli, including chronic predator stress, enhanced cage environments, and high-fat diet (McLeod, Sinal & Perrot-Sinal, 2007; Korgan et al., 2018; Bellisario et al., 2015; Connor et al., 2012).

Offspring from high LG/ABN dams have been demonstrated to have differences in stress reactivity compared to offspring from low LG/ABN mothers (Caldji et al., 1998; Caldji, Diorio & Meaney, 2003). This effect is likely due, in part, to the effects of high LG/ABN behaviours on $GABA_A$ receptor mRNA subunit expression (Caldji, Diorio & Meaney, 2003). Adult rats reared by high LG/ABN dams demonstrated differences in $GABA_A$ receptor subunit expression in the amygdala (Caldji, Diorio & Meaney, 2003). This difference in the composition of $GABA_A$ receptors is thought to be the underlying reason for high LG/ABN offspring showing less fear in novel situations, when compared to offspring from low LG/ABN dams (Caldji et al., 1998). This differential gene expression in the amygdala is thought, in turn, to exert differential effects on CRF gene expression in the hypothalamus, resulting in decreased CRF expression in response to a stressor in high LG/ABN offspring (Caldji, Diorio & Meaney, 2003). Offspring from high LG/ABN dams also exhibit increased numbers of both MR and GR receptors in hippocampal tissue, which has been shown to enhance negative feedback of the stress response (i.e., greater shutting down of subsequent CRF release; Champagne et al., 2008; Meaney & Szyf, 2005). Interestingly, offspring from high LG/ABN dams have improved learning

and memory under baseline conditions, but poorer outcomes when their memory is tested in a stressful situation (Champagne et al., 2008). In contrast, low LG/ABN offspring have poor learning and memory at baseline, but these behaviours are enhanced in response to a stressor (Champagne et al., 2008). This highlights that maternal care programs offspring in an environment-dependent manner, and that a 'match' between early conditions and later environment may be key to understanding the adaptiveness of such programming. Considering the effects of active maternal care, and our finding that probiotics increase active maternal care, we hypothesized that offspring of probiotic-treated dams in the present experiment would be similar to high LG/ABN offspring from other experiments, having for example, differential $GABA_A$ subunit expression and decreased fear/stress reactivity behaviours. We also hypothesized that offspring from probiotic-treated dams would show increased numbers of MRs and GRs in the hippocampus (Caldji, Diorio & Meaney, 2003; Champagne et al., 2008). These ideas will be tested in future studies, with offspring differences in GRs, based on maternal probiotic treatment, being at the top of the list.

In terms of possible mechanisms for the differences observed in maternal care, probiotic treatment, similar to HFD, may act as a stressor, influencing the HPA axis response in dams, to produce altered maternal care behaviour (Barouei et al., 2012). A study by Bellisario and colleagues (2015) hypothesized that HFD would act as a stressor during pregnancy by acting synergistically with the endocrine adaptations characterizing pregnancy and parturition. It was found that maternal HFD increased levels of plasma CORT during the late stages of gestation, compared to control diet (Bellisario et al.,

2015). HFD-fed dams also had lower levels of 11β-HSD-2 enzyme activity, which regulates fetal exposure to maternal glucocorticoids via the placenta (Bellisario et al., 2015). When this enzyme is maintained at high levels, CORT is 5-10-fold higher in mothers than in the fetal samples, because this enzyme inactivates CORT before fetal exposure (Chapman et al., 2013). Interestingly, the dams exposed to HFD also displayed increased time engaging in social interaction (i.e., sniffing) with their pups during a pup retrieval task, meant to determine how quickly a dam returns her pups to the nest (Bellisario et al., 2015). This increased interaction time demonstrates a disorganized behavioural pattern, which is consistent with other findings that HFD disrupts maternal care behaviour (Connor et al., 2012). However, as we know from previous studies examining the effects of stressors on maternal care, stressors can have beneficial effects on maternal care (Korgan et al., 2016; Korgan et al., 2018; McLeod, Sinal & Perrot-Sinal, 2007). For example, it has been found that acute predator odour exposure stress shortly after parturition increases active maternal care (McLeod, Sinal & Perrot-Sinal, 2007). Lastly, it is reasonable to hypothesize that probiotic treatment in females is simply signaling a shift in improved nutrition availability, which affects how the dam interacts with her offspring.

Despite no significant differences between probiotic- and placebo-treated dams concerning their inflammation profiles at postpartum day (PPD 26), when inflammation profiles were correlated with active maternal care behaviours, we found that both ABN and LG were significantly negatively correlated with a number of inflammatory markers. Although there are presently no studies that have examined active maternal care

behaviours and inflammation, immune function and pregnancy/postpartum have been studied extensively. During pregnancy, the body's cell-mediated immune function and Th1 cytokine production are suppressed, and the humoral immunity and Th2 cytokine production are increased (Wilder, 1998). This results in an anti-inflammatory state, designed to protect the growing fetus (Aagard-Tilley et al., 2006; Luppi, 2003; Schumacher et al., 2014; Duarte-Guterman, Leuner & Galea, 2019). Once parturition occurs, the postpartum period is defined by a change to a pro-inflammatory state, which promotes healing throughout the postnatal period (Vannuccini et al., 2016; Groer et al., 2015; Eid et al., 2019). It is unclear whether or not estrogen levels, which are typically low in the postpartum period, affect the pro-inflammatory cytokine levels during this time. A recent study by Eid and colleagues (2019) using a rat model found that 17-βestradiol levels, the main form of estrogen, had no significant effect on postpartum circulating cytokine levels.

In humans, there have been the beginnings of research focused on postpartum effects of probiotics. A placebo-controlled study in women at 14-16 weeks' gestation looked at the effects of *L. rhamnosus* HN001 on postpartum depression and anxiety (Slykerman et al., 2017). It was found that a dose of 6×10^9 CFU starting at recruitment and finishing six months postpartum was sufficient in significantly reducing scores on the Edinburgh Postnatal Depression Scale (EPDS; Cox et al., 1987) and the State-Trait Anxiety Inventory 6 item version (STAI6; Marteau & Bekker, 1992; Slykerman et al., 2017). Due to the quick decrease in postpartum estrogen, it would be of interest to investigate in future studies if *Lactobacillus* strains offer benefits due to altering estrogen and oxytocin

levels postpartum, resulting in more active maternal care in rats, and lower postpartum anxiety in humans (Hendrick, Altshuler & Suri, 1998; Brummelte & Galea, 2016).

To our knowledge, there are no studies to date that have tested whether or not probiotics influence estrogen and oxytocin levels when administered to pregnant or lactating rats. However, based on previous studies demonstrating that products of the gut microbiota, in particular β-glucuronidase, facilitate the deconjugation of estrogen through the removal of heat shock proteins and subsequent binding to ER-α receptors, and the finding that *Lactobacillus* bacteria can increase hypothalamic oxytocin, investigating hormone levels during probiotic treatment postpartum is an interesting avenue for future study (Ljungh & Wadstrom, 2006; Plottel & Blaser, 2011; Poutahidis et al., 2013).

In the present experiment it was found that dams provided the probiotic treatment Lacidofil® had detectable levels of the two probiotic strains contained in Lacidofil® *L. rhamnosus* R0011 and *L. helveticus* R0052 in their caecum samples. Placebo-treated dams showed no detection of these strains in their caecum samples. Although it has previously been hypothesized that probiotics may be able to colonize the newborn gut, we observed that none of the offspring, regardless of maternal treatment condition, showed any detectable level of either probiotic strain in the caecum in adolescence, which is consistent with previous findings (Hashemi et al., 2016, Sarkar et al., 2016; Callaghan, Cowan & Richardson, 2016). It is possible that probiotic strains are transferred to the offspring for the duration of breastfeeding, but that these supplemented

microbes do not colonize the gut in a long-lasting way (Gueimonde et al., 2007; Martin et al., 2009; Fernandez et al., 2013).

Further studies must determine if probiotic supplementation throughout these critical periods has a continuing ability to maintain gut eubiosis throughout life (Hashemi et al., 2016). In particular, long-term studies are necessary to determine if an altered microbiota is the only requirement in developing disease, or if a secondary insult, like psychological stress, diet, or antibiotics, is required to upset the system (Mulligan & Friedman, 2017).

In the present study, adolescent offspring anxiety-like behaviour was assessed using the OFT, one week before acute stress exposure and sacrifice. Our original hypothesis was that offspring from probiotic-treated dams would display fewer anxiety-like behaviours in the OFT, compared to their counterparts from placebo-treated dams, due to differences in maternal care. As stated previously, differences in maternal care, specifically LG/ABN behaviours, can program offspring behaviour (Caldji et al., 1998). However, we found no differences in offspring behaviour based on maternal treatment condition, nor did we observe any sex differences in this test.

These findings are contrary to studies that have also looked at variation in maternal care and offspring behavioural outcomes. Caldji and colleagues (1998) found that offspring from high LG/ABN dams showed a significantly higher amount of exploratory behaviour in the open field, compared to offspring from low LG/ABN dams. Zhang and colleagues (2004) also demonstrated similar results, where offspring from high LG/ABN dams

engaged in more exploration than their low LG/ABN offspring counterparts. Offspring from high LG/ABN dams also showed less fear in a different environment, compared to offspring from low LG/ABN dams (Caldji et al., 1998). Another study by Menard and Hakvoort (2007) found that rat offspring from high LG dams spent less time burying an electrified metal probe in an anxiety-like test called the shock-probe burying test, compared to progeny of low LG dams (De Boer & Koolhaas, 2003; Menard & Hakvoort, 2007). The same rats were then subjected to the resident-intruder paradigm, a task designed to assess defensive behaviours in rodents (Koolhaas et al., 2013). The rats from high LG dams were less aggressive than their counterparts from low LG dams (Menard & Hakvoort, 2007).

Interestingly, Menard and Hakvoort (2007) found in their study that offspring from dams treated with CORT during lactation did not differ from the offspring of placebo-treated dams, despite CORT treatment increasing maternal LG behaviour. This finding is contrary to other studies that have found that treating dams with CORT can reduce stress-related behaviours in the shock-probe test, as well as the resident-intruder test (Casolini et al., 1997; Catalani et al., 2002). It is hypothesized that there may be a critical threshold of maternal care behaviours like LG/ABN necessary for the neural programming of offspring to be observable in tests of anxiety-like, or stress reactivity behaviours (Menard & Hakvoort, 2007; Mashoodh, Sinal & Perrot-Sinal, 2009). The results from the present study are consistent with this theory. Despite the probiotic treatment significantly increasing maternal LG/ABN, compared to the placebo treatment group, the critical threshold needed to elicit the neurobiological changes associated with stress-related or

anxiety-like functions may not have been reached, resulting in no measurable differences in the offspring.

Consistent with expecting decreased anxiety-like behaviour, we had hypothesized that through probiotic administration to mothers, the offspring's stress responding (i.e. behaviours elicited in response to an acute stress and stress hormone/inflammation) would be affected. We hypothesized that offspring from probiotic-treated dams would show lower stress reactivity (i.e. fewer stress-related behaviours and lower ACTH/CORT and inflammation levels) compared to offspring born to placebo-treated dams.

We found no significant effects of maternal treatment, sex, or interaction between the two in the predator odour exposure test. However, we did observe trending effects for portions of the test. We found a main trending effect of maternal treatment in minutes 5-10 of the test with maternal probiotic-treated offspring spending less time rearing and contacting the predator odour stimulus relative to maternal placebo-treated offspring. In the second half of the predator odour test (15 minutes), maternal probiotic-treated offspring continued to show a trending effect where they reared for less time, compared to maternal placebo-treated offspring. Although rearing is often considered an exploratory behaviour, in the context of a stressful environment, there is supporting evidence that rearing may be interpreted as a type of escape behaviour (Lever, Burton & O'Keefe, 2006). In particular, supported rearing (i.e., when the rat has its front limbs touching the sides of the testing apparatus) is thought to be demonstrative of escape-like behaviour, whereas unsupported rearing is primarily exploratory (Lever et al., 2006).

When rats are subjected to an open field test without a hide box, there is an increase in rearing behaviour, compared to an open field test administered without a hide box (Genaro & Schmidek, 2000). In the present study, we did not measure the time of supported versus unsupported rearing separately, so our present finding of more time spent rearing in the placebo-treated offspring must be interpreted with caution. However, it is possible that the offspring from probiotic-treated dams may be demonstrating a less defensive response to the predator odour, compared to their counterparts from placebo-treated dams. Indeed, findings from previous studies using the defensive burying test as a stress-inducing behavioural task have demonstrated that offspring from low LG/ABN dams have a more active approach (i.e., they engage in more burying behaviours to hide the electrified probe) than offspring from high LG/ABN dams (Menard, Champagne & Meaney, 2004; Menard & Hakvoort, 2007; Koolhaas, 2013). The predator odour exposure paradigm has been used in previous studies, demonstrating that compared to a control odour condition, adolescent Long-Evans rats do demonstrate significant defensive behaviours in response to the predator odour (Korgan et al., 2016; Korgan et al., 2018). Other studies using acute predator odour have also demonstrated its ability to elicit defensive behaviours in both sexes (Falconer & Galea, 2003). However, as discussed above, environmental manipulations that affect maternal care are not equal in their ability to elicit programming effects on offspring, which may explain only trending effects between the offspring groups in this study (Menard & Hakvoort, 2007; Mashoodh, Sinal & Perrot-Sinal, 2009).

During the first half (15 minutes) of the predator odour exposure, we found a main trending effect of sex, where male offspring groomed more than female offspring. This finding is consistent with previous work demonstrating that juvenile male rats groomed three times more in response to a novel situation, compared to their juvenile female counterparts (Thor, Harrison, Scheider & Carr, 1988). One explanation for the mechanisms behind this sex-specific difference in grooming is that dams engage in greater amounts of anogenital-directed LG behaviour with their male pups (Moore & Morelli, 1979). This greater amount of LG behaviour for male pups is translated into a differential epigenetic pattern that modifies the expression of ER-α mRNA in the amygdalae of male offspring, which has implications for socioemotional behaviours (Edelmann & Auger, 2011).

Beyond the behavioural stress response, our results from the plasma samples of offspring demonstrated no significant or trending effects of maternal probiotic exposure, sex or an interaction between these two independent variables on ACTH or CORT levels. However, we did observe a trending interaction of sex and maternal treatment on melatonin levels. When this interaction was probed, it was revealed that females from placebo-treated dams had higher melatonin levels than their male counterparts from placebo-treated dams.

Our finding that ACTH levels did not differ based on maternal treatment is understandable, given that ACTH levels return to normal quickly post-stress, whereas corticosterone levels can remain elevated for up to 90 minutes post-stress (Kant, Mougey,

& Meyerhoff, 1989; Ait-Belganoui et al., 2012). A previous study on probiotics and the HPA axis found that the direct probiotic treatment of rats two weeks before an acute stressor lowered ACTH and corticosterone levels (Ait-Belgnaoui et al., 2012). Our present findings are inconsistent, however, because the rat offspring in this study did not directly receive the probiotic, but instead had contact with the treatment throughout gestation, and while they nursed from their mother. It is possible that probiotic treatment from the mother only elicits a small effect, via its effects on maternal care, on the programming of the offspring's stress response and does not affect ACTH or CORT release in a significant way.

Another possible explanation for these results is the time course from the start of stress exposure, to the time of sacrifice. ACTH and corticosterone levels fluctuate based on the animal's circadian rhythms, and it has been found that an animal exposed to a stressor during a circadian high point will have higher levels of corticosterone release in response to stress, compared to the magnitude of release when the animal is at the nadir of the cycle (Windle, Wood, Shanks, Lightman, & Ingram, 1998). Based on other studies that utilized different forms of mild to moderate acute stressors (e.g. tail nick or restraint), CORT levels reach their highest concentrations approximately 20-30 minutes into stress exposure, and return close to baseline by 40 minutes post-exposure (Weibel, Maccari &Van Reeth, 2002; Garcia et al., 2000).

Our finding that melatonin levels were higher in female offspring from placebo-treated dams compared to their male counterparts is interesting, as melatonin is released from the

mammalian pineal gland, which can differentially synthesize melatonin, based on sex hormone exposure (Ekmekcioglu, 2006; Luboshitzky et al., 1997). For example, studies in rats have found that exogenous estradiol administration stimulates pineal parenchymal cells, thus increasing pineal gland activity (Cardinali et al., 1987; Sinha, Chattopadhyay & Chakraborty, 2010). Exogenous testosterone administration has an opposing effect, inhibiting the cellular processes of the pineal gland (Cardinali et al., 1987; Sinha, Chattopadhyay & Chakraborty, 2010). Similarly, in humans, it has been found in healthy young adults that females had higher melatonin levels across a 32-hour period, compared to their male counterparts (Gunn et al., 2016). Based on these previous findings, we expected in the present study to observe that all females, regardless of maternal treatment, would have higher melatonin levels. However, in a maternal separation (MS) model in rats, it was demonstrated that Lacidofil® administration to dams during the separation period (PD 2-14) corrected the abnormal pubertal timing that typically occurs in MS models, in both male and female adolescent offspring (Cowan & Richardson, 2018). While the effects of probiotic administration have not been studied in standard-reared adolescents, it is possible that the probiotic treatment to dams affects estrogen levels in their female offspring, thereby affecting melatonin secretion (Cowan & Richardson, 2018).

We found that males from probiotic-treated dams had a trend towards lower plasma levels of the pro-inflammatory analyte, GRO/KC, compared to males raised by placebo-treated dams. GRO/KC, also known as CXCL-1, has similar characteristics to early acute inflammatory phase proteins (Campbell et al., 2003). GRO/KC is released in response to

acute injury, and, interestingly, levels increase in proportion to injury severity (Campbell et al., 2003). Due to the acute nature of the stressor to which animals were exposed in the present study, the expression of GRO/KC as an acute inflammatory response is understandable, as acute psychological stressors (i.e., predator odour) have been found to evoke the sterile inflammation response through increasing the circulating levels of damage-associated molecular patterns (DAMPs) in the blood (Fleshner, 2004; Fleshner, Frank & Maier, 2017). The sterile inflammation response is initiated through the recruitment of DAMPs, which bind to pattern recognition receptors (PRRs) to initiate the cascading effects of inflammatory markers, such as cytokines (Fleshner, Frank & Maier, 2017). The reason that we only observed this trending effect for one of the 23 inflammatory analytes measured in plasma could be due to differences in timing associated with the release of inflammatory markers. For example, the time course for measuring GRO/KC has been reported as approximately 2 hours post-stressor, which coincides well with our 1.5-hour post-stressor onset collection time (Campbell et al., 2003). Similar to the present study, Ait-Belganoui and colleagues (2012) found no differences in the inflammatory markers IL-1β, IL-6 and TNF-α, when plasma levels were measured 2 hours post-stress exposure. Our trending finding for GRO/KC was only found in male rats and this may be due to a possible protective effect of estradiol on inflammation (Pyter et al., 2013). Pyter and colleagues (2013) observed that when female rats were injected with the acute endotoxin, LPS, this increased their circulating levels of estradiol. In both *in vitro* and *in vivo* experiments, LPS has been shown to increase levels of GRO/KC, but sex differences have not been assessed to date (Li et al., 2009; Shibata et al., 1996). However, if estradiol did elicit protective effects on inflammatory markers

such as GRO/KC, we would have expected to observe lower amounts of this analyte in the female rats, which is not what we observed. A review of animal and human studies involving circulating testosterone levels in males found that low testosterone predisposes males to systemic inflammation, demonstrating the testosterone may, like estradiol, have a protective effect against inflammation (Mohamad et al., 2019).

We also observed many significant, negative correlations between plasma CORT levels and plasma inflammation levels in the offspring from probiotic-treated dams, but not in the offspring from placebo-treated dams. We hypothesize that this difference may be due to the differences in maternal care provided to each of these offspring groups. The offspring from probiotic-treated dams may have increased levels of GRs, which, as previously stated, occurs in offspring that receive higher levels of LG and ABN (Meaney & Szyf, 2005). The enhanced sensitivity to glucocorticoids released during an acute stressor that results from increased numbers of GRs in the brain is thought to improve control over the expression of inflammation (Bauer & Teixeira, 2018). Thus, when GRs are activated by a stressor, they are able to reduce the expression of inflammation signalling pathways, such as NF-κB (Bauer & Teixeira, 2018).

4.2 Limitations & Future directions

We found that dams treated with Lacidofil® engaged in significantly more arched back nursing, compared to their placebo-treated counterparts. One limitation to our maternal care observations is that due to the nature of having separate, clearly marked colony rooms to reduce cross-contamination and utilizing a live-scoring method, two trained observers had to be employed for these behavioural observations and were unable to be

blinded. In future studies, we will consider using a less time-consuming, but equally effective measure of maternal care, as used in previous studies, so that the same experimenter can observe both placebo- and probiotic-treated dams and we can include duration, as well as frequency, of active maternal behaviours (McLeod, Sinal, Perrot-Sinal, 2007; Mashoodh, Sinal & Perrot-Sinal, 2009). Our future experiments will also consider the use of a molecular marker to investigate the underlying mechanisms of maternal care, such as ER-α receptors, which would help confirm if probiotic treatment in dams is altering the receptor composition of the mPOA (Champagne et al., 2003b).

Despite our finding that probiotic-treated dams engaged in significantly more arched back nursing and had a trend toward higher licking/grooming, both known for the ability to reduce offspring anxiety-like behaviours, we found no differences between maternal treatment in the offspring's behaviour in the OFT. It is possible that because we only utilized one measure of anxiety-like behaviour, that this single test on its own was not sufficient to detect any differences present due to maternal treatment. There is a consensus in the behavioural neuroscience community that the popular tests for anxiety-like behaviours (e.g. OFT, EPM, LDB) are not ideal, as these tests do not have a common anxiety factor (O'Leary, Gunn & Brown, 2013; Ramos et al., 1998; Ramos, 2008). In order to gain a more complete idea of the general anxiety-like behaviours in our rat model, future studies should seek to incorporate multiple behavioural measures of anxiety to improve the comprehensiveness of this behavioural model (O'Leary, Gunn & Brown, 2013; Ramos, 2008).

Lastly, the timepoints used for the measurement of both stress hormones and inflammatory markers was a significant limitation in this study. We wanted to investigate the effects of maternal treatment on the programming of offspring stress responding and acute inflammation, since the nature of the effects of acute stress exposure on inflammation is not well understood. There is evidence that low levels of glucocorticoids result in enhanced immunity, whereas high levels of circulating glucocorticoids result in suppressed immunity (Bauer & Teixeira, 2018; Cain & Cidlowski, 2017). In order to understand the magnitude of our acute psychological stressor's effect on stress hormone levels and inflammation markers, baseline levels of these compounds would need to be known. However, our experimental design did not allow for this, and so we had to do our best to capture both the stress hormone peak, which may continue for up to one-hour post stress exposure, and the onset of inflammatory marker proteins (approximately 2 hours after initial exposure). Ait-Belgnaoui and colleagues (2012) found differences in inflammatory markers at 2 hours post-stress exposure, however, they detected differences in mRNA, which would precede the proteins themselves.

4.3 Implications & Concluding remarks

The present study demonstrates that probiotic treatment, Lacidofil®, has the ability to influence active maternal care behaviours that are crucial in the development of offspring. This finding has implications for how probiotics interact with a pregnant or lactating host, and how this may affect hormone concentrations, or how hormones act in the body. We provide evidence that offspring exposed to probiotic treatment throughout pregnancy and lactation are heavier at weaning than their placebo-exposed counterparts, which warrants further investigation of how probiotics affect maternal milk composition,

and nutrient intake in young offspring. Although we were unable to find any significant programming effects of maternal probiotic treatment on offspring stress outcomes, there may be subtle effects at work here, such as changes in GR concentration in the brain, that will have long-term impacts on offspring stress resiliency.

In sum, this thesis aimed to demonstrate the potential early life programming power of Lacidofil®, and to further the knowledge of probiotics and their application in behavioural neuroscience studies. The gut-brain axis has been strongly implicated in stress-related disorders, and there are many implications for understanding not only how this axis functions, but how we can ameliorate stress-related outcomes that occur when the gut-brain axis is disrupted.

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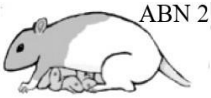
APPENDIX A MATERNAL CARE BEHAVIOUR ETHOGRAM

Maternal Care Observations Ethogram
Perrot Lab (Adapted from Champagne et al., 2003b)

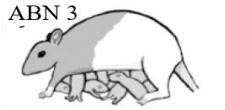
Passive Nursing (PN): Lying on side nursing pups, but not in an arched back nursing posture

Blanket Posture (BP): Lying prone on top of pups

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



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



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

Licking/grooming (LG): Usually oral or anogenital, and can occur in conjunction with arched-back nursing

Separated pups (SP): Pups found away from the litter and/or outside the nest

Pup retrieval (PR): Dam carrying pups towards the nest

Pup move (PM): Dam observed carrying pups away from the nest

No contact with pups (NCP): Dam is not in contact (passive or otherwise) with her litter

Feeding (F): Dam is eating or drinking

Nest building (NB): Dam is moving cage bedding around with her feet or nose.

Self-grooming (SG): Dam is using her paws or mouth to comb, lick or bite at her fur.

Pup contact (PC): Dam is in contact with her pups, not including blanket-posture nursing, arched-back nursing, passive nursing, or licking/grooming

APPENDIX B OPEN FIELD TEST SCORING SHEET

Open Field Test (OFT) Scoring Sheet
Observer ID: _____
Project ID: _____

Perrot Lab
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Rat/Video ID:	0 - 30 seconds	30 sec. - 1 min.	1 - 1.5 minutes	1.5 - 2 minutes	2-4 minutes	4-6 minutes	6-8 minutes	8-10 minutes
Frequency of Line Crosses								
Frequency of Rearing								
Frequency of Freezing								
Thigmotaxis (seconds)								
Center Time (seconds)								
Initial Latency to Move (seconds)								

APPENDIX C OPEN FIELD TEST ETHOGRAM

Open Field Test (OFT) Ethogram

1. The frequency of line crosses: The number of times the rat moves over one of the quadrant lines in the open field maze (all paws must cross the line).

2. The frequency of rearing: The number of discrete times the rat is standing on hind-paws, while the animal may or may not be leaning on a perimeter wall.

3. The frequency of freezing: A tense, crouching-like posture, absent of all movements except those associated with breathing and the increased muscle tone from maintaining this tense pose (Roelofs, 2017).

4. Thigmotaxis: The propensity of the rat to remain in close contact with the walls of a space; so, the duration of time (in seconds) will be recorded when any part of the animal's body (except the tail and vibrissae) is in contact with one of the four walls of the arena (Joshi, Leslie & Perrot, 2017).

5. Centre Time: The duration of time (in seconds) that the rat spends in the centre four squares (all paws must be within the boundary lines for these four squares).

6. Initial Latency to Move: The duration of time (in seconds) that elapses from the moment the rat is placed in the maze until the rat moves outside of the centre area (designated by the four inside squares).

APPENDIX D PREDATOR ODOUR EXPOSURE SCORING SHEET

Predator Odour Exposure (POE) Scoring Sheet
Observer ID: _____
Project ID: _____

Animal/Video ID:	0-5 minutes	6-10 minutes	11-15 minutes	16-20 minutes	21-25 minutes	26-30 minutes
Frequency of Supported Rearing						
Frequency of Unsupported Rearing						
Duration of Rearing (seconds)						
Frequency of Stimulus Contacts						
Duration of Vicinity Time (seconds)						
Frequency of Self-Grooming						
Frequency of Freezing						

APPENDIX E PREDATOR ODOUR EXPOSURE ETHOGRAM

Predator Odour Exposure (POE) Ethogram

1. The frequency of supported rearing: The number of discrete times the rat is standing, with an extended body posture on their hind-paws, while the animal has one, or both paws touching a perimeter wall. The animal must touch down with the front feet then extend again for these behaviours to be counted as two separate rearing events.
    a. Note. A behaviour does NOT count as rearing if the rat is behind the stimulus line (marked with tape) and facing the stimulus.
2. The frequency of unsupported rearing: The number of discrete times the rat is standing, with an extended body posture on their hind-paws, while the animal is not touching the perimeter walls. The animal must touch down with the front feet then extend again for these behaviours to be counted as two separate rearing events.
    a. Note. A behaviour does NOT count as rearing if the rat is behind the stimulus line (marked with tape) and facing the stimulus.
3. The duration of rearing: Total time (in seconds) spent standing on hind-paws only while front paws may or may not be touching the perimeter walls.
4. The frequency of self-grooming: The number of times a rat engages in a bout of licking, nibbling, and combing-type actions of the fur lasting longer than 2 seconds (Korgan et al., 2016).
5. The duration of odour stimulus vicinity time: The amount of time (in seconds) spent within 10 cm of the wall where the odour stimulus was positioned (Korgan et al., 2016). This is denoted by a piece of white or black tape on the wall of the odour exposure arena. The rat should be rearing (unsupported, or supported) on, or facing, the wall containing the odour stimulus (approximately 1-centimetre piece of cat collar), with the nose pointed in the direction of the odour stimulus.
6. The frequency of odour stimulus contacts: The number of discrete times that the animal reared up to approach and/or contact the odour stimulus (Korgan et al., 2016).
7. The frequency of freezing: A tense, crouching-like posture, absent of all movements except those associated with breathing and the increased muscle tone from maintaining this tense pose (Roelofs, 2017).

Table 1 Descriptions of the first 13 out of 23 inflammation markers analyzed in dam and offspring plasma samples, including their associated cellular pathways and their functions, as adapted from the Rat Genome Database (RGD; Shimoyama et al., 2014)

Analyte	Cellular Pathway Participation[170]	Brief Description of RGD Function[170]
Interleukin-1α (IL-1α)	IL-1 signaling pathway; Apoptotic cell death pathway; Cytokine mediated signaling pathway	Interleukin-1 receptor binding activity, regulates intracellular signalling
Interleukin-1β (IL-1β)	IL-1, 12 and 23 signaling pathways	Involved in response to vitamins, regulates nervous system proliferation
Interleukin-2 (IL-2)	IL-2, 12, and 23 signaling pathways	Involved in carbohydrate binding, regulates protein phosphorylation
Interleukin-4 (IL-4)	Fc epsilon receptor mediated signaling pathway; IL-4 and 12 signaling pathways	Involved in regulating leukocyte migration and regulating the host defence response
Interleukin-5 (IL-5)	Fc epsilon receptor mediated signaling pathway; IL-4 and 5 signaling pathways	Involved in cytokine activities and interleukin-5 receptor binding activity
Interleukin-6 (IL-6)	IL-6, 23, and 27 signaling pathways	Involved in interleukin-6 receptor binding activity and regulating cellular communication
Interleukin-7 (IL-7)	Cytokine mediated signaling pathway; Jak-Stat signaling pathway	Hypothesized to participate in growth factor activity
Interleukin-10 (IL-10)	IL-4 and 10 signaling pathways; Allograft rejection pathway	Involved with cellular aging and the cellular response to estradiol exposure
Interleukin-12p70 (IL-12p70)*	IL-12 and 27 signaling pathways; Allograft rejection pathway	Involved in cell growth, hypothesized to participate in growth factor activity
Interleukin-13 (IL-13)	Cytokine mediated signaling pathway; Fc epsilon receptor mediated signaling pathway; Asthma pathway	Involved in regulating cell growth, regulating metabolic processes
Interleukin-17A (IL-17A)	No known pathway associations	Involved in the cellular response to glucocorticoids, regulating cell death
Interleukin-18 (IL-18)	IL-12, 23, and 27 signaling pathways	Involved in regulating cell proliferation, regulating cytokine production
Granulocyte Colony-Stimulating Factor (G-CSF)	Cytokine mediated signalling pathway; Jak-Stat signaling pathway; Malaria pathway	Shows growth factor activity, regulates neuronal death

*IL-12p70 is the combination of two IL-12 subunits: the IL-12α (p35) and IL-β (p40) subunits.

Table 2 Descriptions of the remaining 10 out of 23 inflammation markers analyzed in dam and offspring plasma samples, including their associated cellular pathways and their function, as adapted from the Rat Genome Database (RGD; Shimoyama et al., 2014)

Analyte	**Cellular Pathway Participation[170]**	**Brief Description of RGD Function[170]**
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)	Fc epsilon receptor mediated signaling pathway; Granulocyte -macrophage colony-stimulating factor signaling pathway; Syndecan signaling pathway	Involved in dendritic cell differentiation, involved in the cellular response to endotoxins
Growth Related Oncogene/Keratinocyte Chemoattractant (GRO/KC)	IL-23 signaling pathway; Chemokine mediated signaling pathway; NOD-like receptor signaling path	Shows chemokine activity, involved in neutrophil chemotaxis
Interferon- γ (IFN-γ)	IL-2, 12, and 23 signaling pathways	Involved in the regulation of cell growth, and neurogenesis
Macrophage Colony Stimulating Factor (M-CSF)	Cytokine mediated signaling pathway; Rheumatoid arthritis pathway	Involved in the regulation of cell growth, and leukocyte differentiation
Macrophage Inflammatory Protein 1α (MIP-1α)	IL-12 signaling pathway; Chagas disease pathway; Chemokine mediated signaling pathway	Involved in neutrophil chemotaxis and the regulation of calcium ion concentration in cytosol
Macrophage Inflammatory Protein 3α (MIP-3α)	Chemokine mediated signaling pathway; Cytokine mediated signaling pathway; Rheumatoid arthritis pathway	Involved in cellular response to bacterial molecules, and healing
Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES)	Syndecan signaling pathway; Chagas disease pathway; Chemokine mediated signaling pathway	Involved with heparin binding and lymphocyte migrating
Tumor Necrosis Factor α (TNF-α)	TNF mediated signaling pathway; Ceramide signaling pathway; Fc epsilon receptor mediated signaling pathway	Involved with cellular communication, and regulating cytokine production
Vascular Endothelial Growth Factor (VEGF)	Vascular endothelial growth factor signaling pathway; Altered vascular endothelial growth factor	Involved with both protein and receptor ligand activities
Monocyte Chemoattractant Protein (MCP-1)	Angiotensin II signaling pathway; Vascular endothelial growth factor signaling pathway; GM-CSF signaling pathway	Involved with aging, organ regeneration, calcium ion homeostasis

Table 3 Descriptive statistics for all litters at birth (PD 0)

Dam ID	Treatment	Litter Size	Birth Survival Ratio	Male Ratio	Birth Weight (g) M (SD)	Male Birth Weight (g) M (SD)	Female Birth Weight (g) M (SD)
M	Placebo	17	0.94	0.56	6.07	6.18	5.93
N	Placebo	13	1.00	0.38	6.40	6.46	6.36
O	Placebo	11	1.00	0.64	6.15	6.37	5.78
Q	Placebo	12	1.00	0.42	5.91	6.08	5.79
R	Placebo	18	1.00	0.61	5.71	5.83	5.53
S	Placebo	11	1.00	0.45	6.62	6.86	6.42
T	Placebo	15	1.00	0.60	5.36	5.46	5.22
I	Probiotic	14	0.93	0.38	6.28	6.48	6.12
J	Probiotic	13	1.00	0.15	5.49	5.55	5.48
K	Probiotic	7	1.00	0.43	7.89	8.23	7.63
U	Probiotic	15	1.00	0.40	5.35	5.43	5.29
V	Probiotic	12	0.83	0.67	6.61	6.69	6.45
W	Probiotic	8	1.00	0.63	7.10	7.22	6.90
X	Probiotic	12	1.00	0.75	6.00	6.05	5.87
Y	Probiotic	11	1.00	0.64	6.79	6.94	6.40

Table 4 Effects of probiotic treatment on the frequency of maternal care behaviours from Mann-Whitney U tests.

	Placebo Mdn (Range)	**Probiotic Mdn (Range)**	**U value**	**Sig.**	**Effect Size**
PN	36.00 (140)	40.50 (53)	31.00	$p = .779$	$r = .09$
BP	86.00 (100)	44.00 (110)	23.00	$p = .613$	$r = -.15$
ABN	157.00 (87)	229.00 (64)	52.00	$p = .004$**	$r = .72$
LG	57.00 (35)	83.50 (67)	46.50	$p = .029$[a]	$r = .55$
FS	106.00 (71)	121.00 (65)	46.50	$p = .029$[a]	$r = .55$
NB	21.00 (22)	12.00 (27)	17.50	$p = .232$	$r = -.31$
NCP	213.00 (106)	157.50 (163)	15.00	$p = .152$	$r = -.39$
PC	29.00 (24)	34.50 (51)	33.50	$p = .536$	$r = .16$
SP	2.00 (39)	0.50 (2)	15.00	$p = .152$	$r = -.41$
PR	0 (3)	0 (11)	27.00	$p = .955$	$r = -.03$
SG	74.00 (60)	57.50 (77)	24.50	$p = .694$	$r = -.10$

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

Table 5 Descriptive statistics including sample sizes, means and standard deviations for offspring anxiety-like behaviours.

Sex	Treatment	*n*	Frequency of Line Crosses M (SD)	Frequency of Rearing M (SD)	Thigmotaxis (seconds) M (SD)	Center Time (seconds)[a] M (SD)	Latency to Move (seconds)[a] M (SD)
Female	Placebo	14	129.43 (52.81)	30.57 (12.70)	200.94 (109.27)	39.46 (23.86)	4.66 (4.21)
	Probiotic	16	105.06 (43.87)	28.13 (12.81)	202.32 (119.49)	30.50 (22.39)	6.33 (5.30)
	Total	30	116.43 (48.97)	29.27 (12.60)	201.68 (112.86)	34.68 (23.13)	5.55 (4.82)
Male	Placebo	14	103.64 (44.82)	24.71 (11.84)	179.86 (86.35)	20.58 (16.35)	4.46 (4.49)
	Probiotic	16	115.63 (48.42)	25.25 (10.59)	229.81 (75.19)	35.22 (29.41)	4.36 (3.66)
	Total	30	110.03 (46.37)	25.00 (10.99)	206.50 (83.12)	28.66 (25.16)	4.41 (4.01)
Both	Placebo	28	116.54 (49.82)	27.64 (12.41)	190.40 (97.23)	30.37 (22.37)	4.56 (4.27)
	Probiotic	32	110.34 (45.77)	26.69 (11.65)	216.07 (99.20)	32.86 (25.82)	5.38 (4.62)
	Total	60	113.23 (47.39)	27.13 (11.92)	204.09 (98.30)	31.72 (24.13)	4.99 (4.44)

[a]One extreme value (three times the interquartile range) was removed prior to statistical tests.

Table 6 Two-way ANOVA results for offspring anxiety-like behaviours measured in the open field test.

Anxiety-Like Behaviour	Variable	F value	Sig.	Effect Size
Frequency of Line Crosses	Sex	.383	$p = .538$	$\eta_p^2 = .007$
	Treatment	.254	$p = .616$	$\eta_p^2 = .005$
	Sex*Treatment	2.185	$p = .145$	$\eta_p^2 = .038$
Frequency of Rearing	Sex	1.978	$p = .165$	$\eta_p^2 = .034$
	Treatment	.095	$p = .759$	$\eta_p^2 = .002$
	Sex*Treatment	.231	$p = .633$	$\eta_p^2 = .004$
Thigmotaxis (seconds)	Sex	.016	$p = .901$	$\eta_p^2 = .000$
	Treatment	1.000	$p = .322$	$\eta_p^2 = .018$
	Sex*Treatment	.895	$p = .348$	$\eta_p^2 = .016$
Center Time (seconds)	Sex	1.297	$p = .260$	$\eta_p^2 = .023$
	Treatment	.208	$p = .650$	$\eta_p^2 = .004$
	Sex*Treatment	3.605	$p = .063$	$\eta_p^2 = .062$
Latency to Move (seconds)	Sex	.870	$p = .355$	$\eta_p^2 = .016$
	Treatment	.448	$p = .506$	$\eta_p^2 = .008$
	Sex*Treatment	.577	$p = .451$	$\eta_p^2 = .010$

Table 7 Descriptive statistics including means and standard deviations for stress hormone concentrations found in offspring plasma and hypothalamus samples (pg/µg) samples normalized by total protein concentration.

Sample Type	**Sex**	**Treatment**	**pg/mL ACTH M (SD)**		**ng/mL CORT M (SD)**		**pg/mL Melatonin M (SD)**	
			M	*SD*	*M*	*SD*	*M*	*SD*
Plasma	Female	Placebo	41.18	26.68	176.30	129.20	163.75	179.44
		Probiotic	35.65	28.42	133.02	951.23	75.15	65.33
		Both	38.23	27.29	152.43	111.72	113.67	132.35
	Male	Placebo	46.43	29.54	117.86	86.28	58.54	95.07
		Probiotic	25.60	19.53	178.56	84.64	134.90	118.01
		Both	35.65	26.61	148.21	89.38	95.06	111.24
	Both	Placebo	43.80	27.75	146.00	110.96	106.37	146.32
		Probiotic	30.79	24.66	154.28	91.79	102.54	95.95
Sample Type	**Sex**	**Treatment**	**pg/µg ACTH M (SD)**		**pg/µg CORT M (SD)**		**pg/µg Melatonin M (SD)**	
Hypothalamus	Female	Placebo	.019	.014	5.06	6.24	.044	.046
		Probiotic	.022	.017	4.93	8.50	.028	.053
		Both	.021	.015	4.99	7.40	.035	.049
	Male	Placebo	.012	.011	6.21	7.45	.041	.035
		Probiotic	.016	.015	4.16	4.78	.033	.030
		Both	.014	.013	5.15	6.19	.037	.032
	Both	Placebo	.016	.013	5.64	6.77	.042	.040
		Probiotic	.019	.016	4.56	6.85	.031	.043

Table 8 Two-way ANOVA results for stress hormone concentrations found in offspring plasma and hypothalamus samples.

	Stress Hormone	Variable	F value	Sig.	Effect Size
Plasma	ACTH	Sex	.123	$p = .728$	$\eta_p^2 = .002$
		Treatment	3.692	$p = .060$	$\eta_p^2 = .063$
		Sex*Treatment	1.242	$p = .270$	$\eta_p^2 = .022$
	CORT	Sex	.059	$p = .808$	$\eta_p^2 = .001$
		Treatment	.108	$p = .743$	$\eta_p^2 = .002$
		Sex*Treatment	3.860	$p = .055$	$\eta_p^2 = .068$
	Melatonin	Sex	.426	$p = .517$	$\eta_p^2 = .010$
		Treatment	.031	$p = .861$	$\eta_p^2 = .001$
		Sex*Treatment	5.614	$p = .022$[a]	$\eta_p^2 = .118$
Hypothalamus	ACTH	Sex	2.675	$p = .108$	$\eta_p^2 = .046$
		Treatment	.747	$p = .391$	$\eta_p^2 = .013$
		Sex*Treatment	.008	$p = .931$	$\eta_p^2 = .000$
	CORT	Sex	.011	$p = .918$	$\eta_p^2 = .012$
		Treatment	.364	$p = .549$	$\eta_p^2 = .007$
		Sex*Treatment	.283	$p = .597$	$\eta_p^2 = .005$
	Melatonin	Sex	.012	$p = .911$	$\eta_p^2 = .000$
		Treatment	1.158	$p = .287$	$\eta_p^2 = .021$
		Sex*Treatment	.088	$p = .767$	$\eta_p^2 = .002$

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

Table 9 Table of Spearman's correlations between offspring stress hormone levels in plasma samples and offspring inflammation markers in plasma samples, based on maternal treatment condition.

Inflammation Analytes (pg/mL)	**Stress Hormone Analytes (pg/mL)**					
	ACTH		Corticosterone		Melatonin	
	Placebo	Probiotic	Placebo	Probiotic	Placebo	Probiotic
G-CSF	.146	.268	-.159	-.386*	-.323	-.329
GM-CSF	.516**	.636**	-.600**	-.727**	-.700**	-.765**
GRO/KC	.501**	.663**	-.644**	-.758**	-.734**	-.762**
IFN- γ	.141	.425*	-.186	-.533**	-.344	-.458**
IL-1α	.358	.396*	-.315	-.705**	-.425*	-.611**
IL-1β	.543**	.631**	-.619**	-.744**	-.700**	-.730**
IL-2	.163	.417*	-.234	-.290	-.367	-.402*
IL-4	.308	.406*	-.210	-.657**	-.390*	-.613**
IL-5	.284	.454*	-.200	-.635**	-.360	-.602**
IL-6	.127	.176	-.103	-.380*	-.297	-.249
IL-7	.548**	.620**	-.642**	-.685**	-.693**	-.700**
IL-10	.299	.470**	-.228	-.638**	-.402*	-.607**
IL-12p70	.389*	.544**	-.329	-.671**	-.447*	-.675**
IL-13	-.027	.148	-.154	-.165	-.370	-.058
IL-17	.358	.503**	-.313	-.586**	-.453*	-.575**
IL-18	.487**	.681**	-.621**	-.547**	-.645**	-.658**
M-CSF	.104	.445*	-.199	-.373*	-.306	-.458**
MCP-1	.608**	.757**	-.726**	-.846**	-.819**	-.870**
MIP-1α	.561**	.552**	-.589**	-.760**	-.674**	-.745**
MIP-3α	.056	.557**	-.249	-.498**	-.409*	-.582**
RANTES	.293	.264	-.213	-.507**	-.388*	-.419*
TNF-α	.149	.364*	-.160	-.485**	-.344	-.378*
VEGF	.488**	.673**	-.588**	-.750**	-.703**	-.779**

* is trending at the $p < .05$ level.
** is significant at the $p < .01$ level.

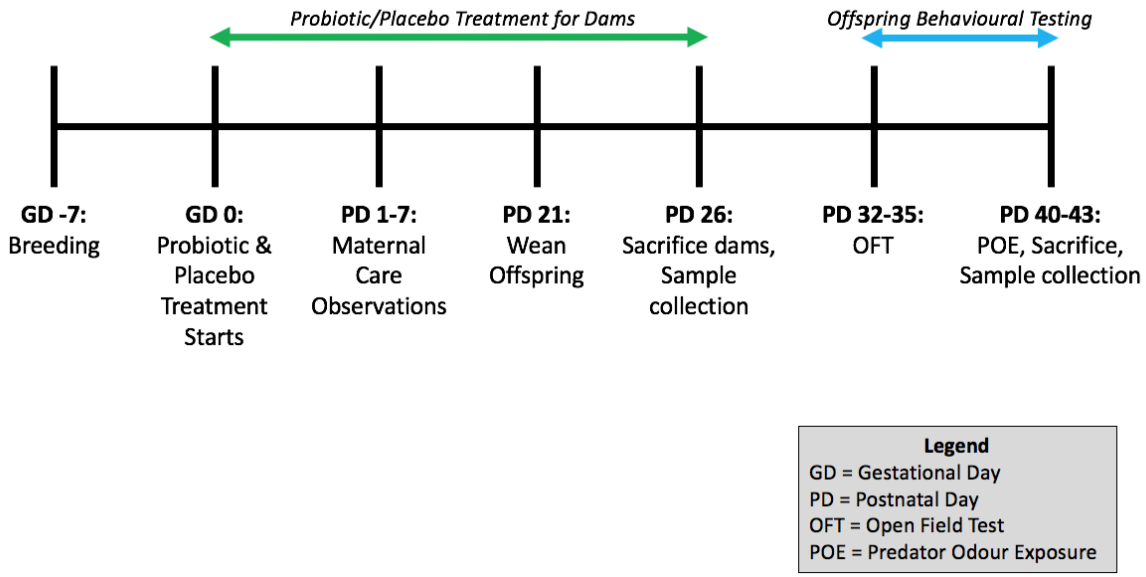

Figure 1 Experiment timeline from breeding to offspring sacrifices.

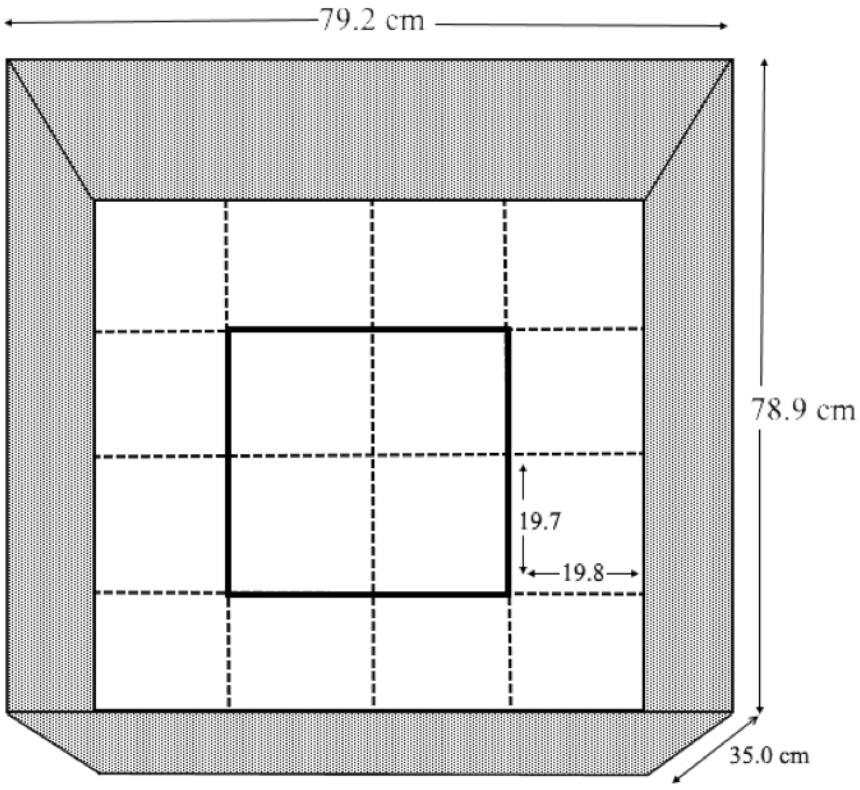

Figure 2 Diagram of the open field arena used to assess offspring anxiety-like behaviour in this experiment.

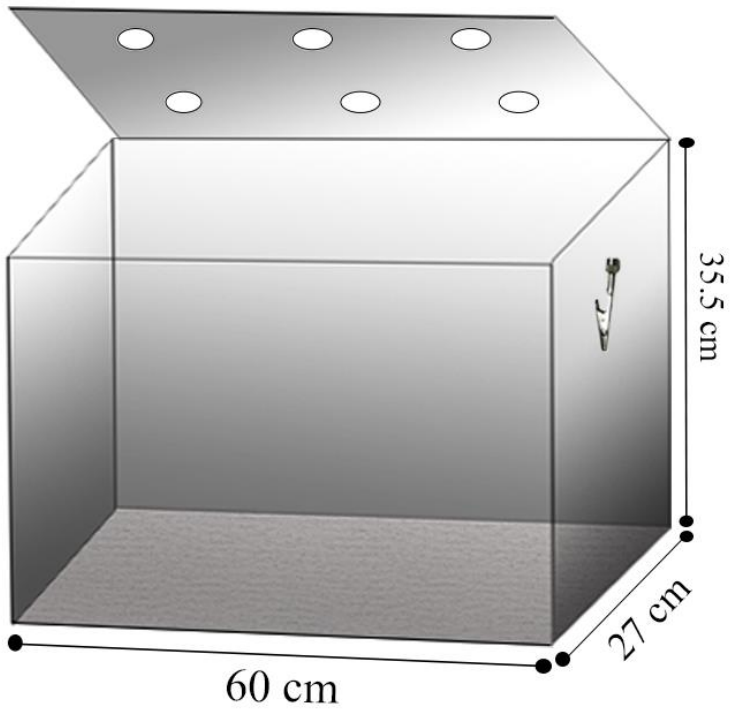

Figure 3 Diagram of the predator odour exposure arena used for the acute stress paradigm performed with offspring in this experiment.

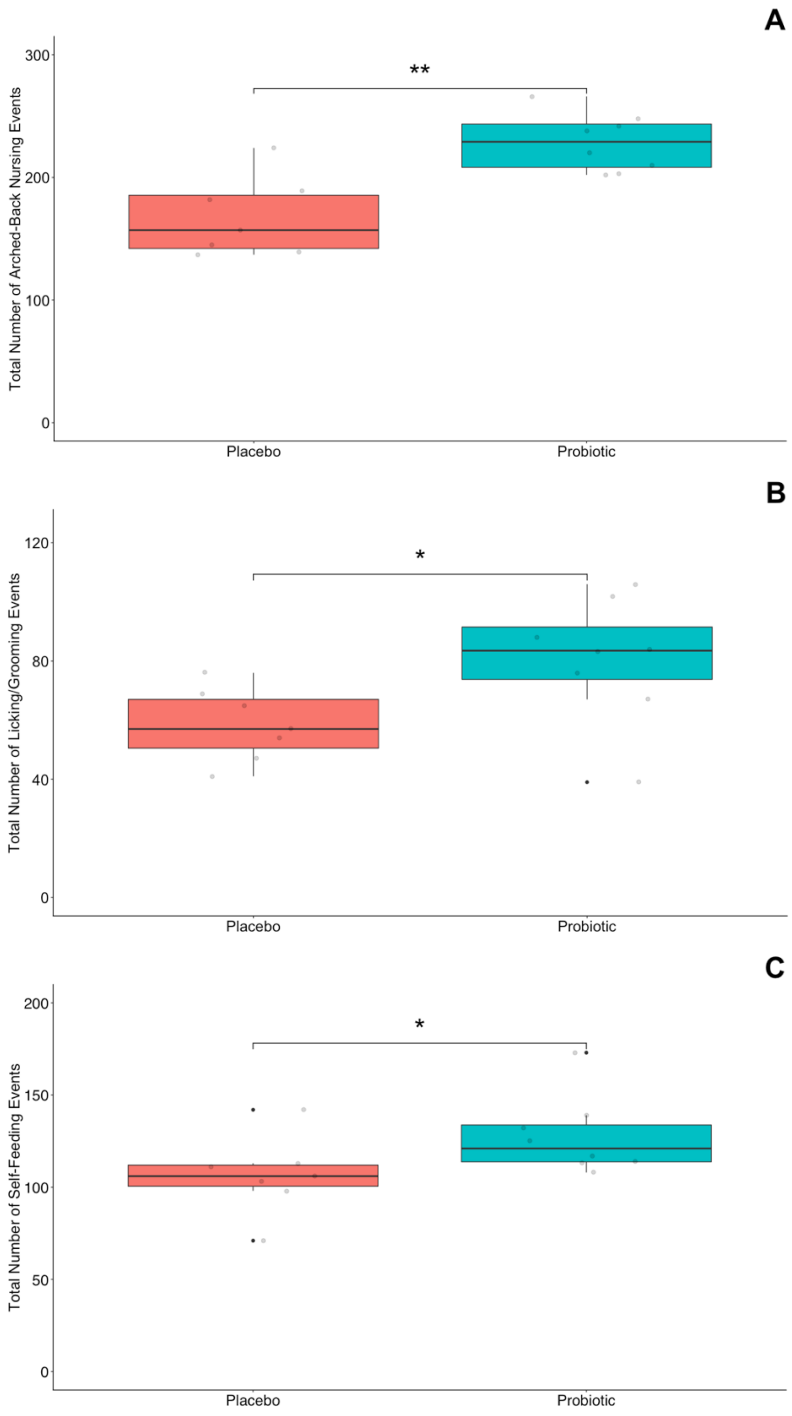

Figure 4 The total number of events recorded across the first seven postnatal days (PD 1-7), after birth on PD 0 for: A) arched-back nursing, B) licking/grooming and C) self-feeding. The median is represented by the thick, black horizontal line, the whiskers represent 1.5 times the interquartile range (1.5 x IQR). The individual transparent dots represent each dam's raw data for total number of events. The solid black dots indicate outliers.

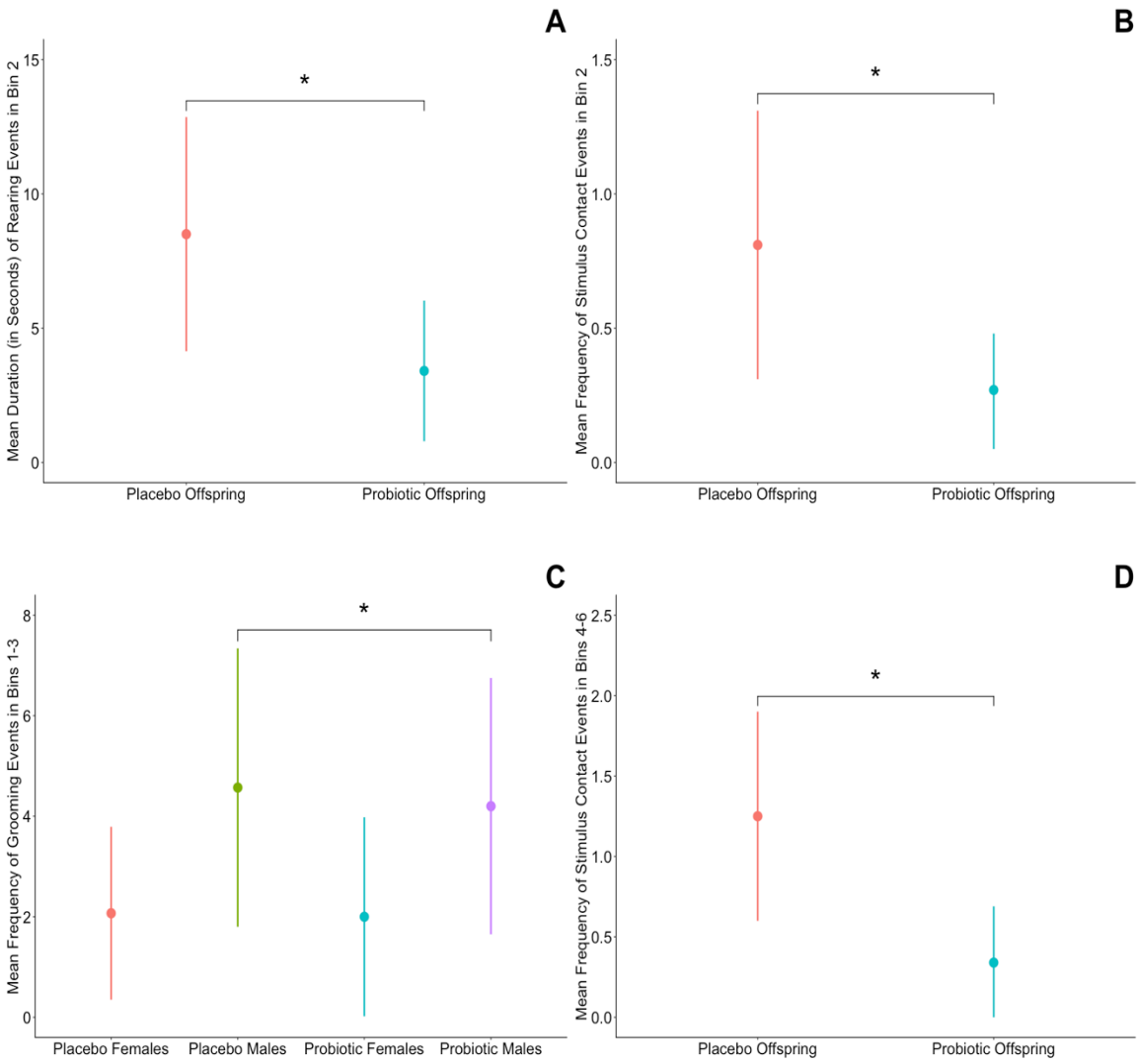

Figure 5 The means of behaviours recorded during offspring predator odour exposure, indicated by the large dot, with error bars representing the standard deviations for: A) Duration in seconds of rearing during bin 2 (minutes 5-10), B) Frequency of stimulus contact events in bin 2 (minutes 5-10), C) Frequency of grooming events in bins 1-3 (minutes 0-15), and D) mean frequency of stimulus contact events in bins 4-6 (minutes 16-30).

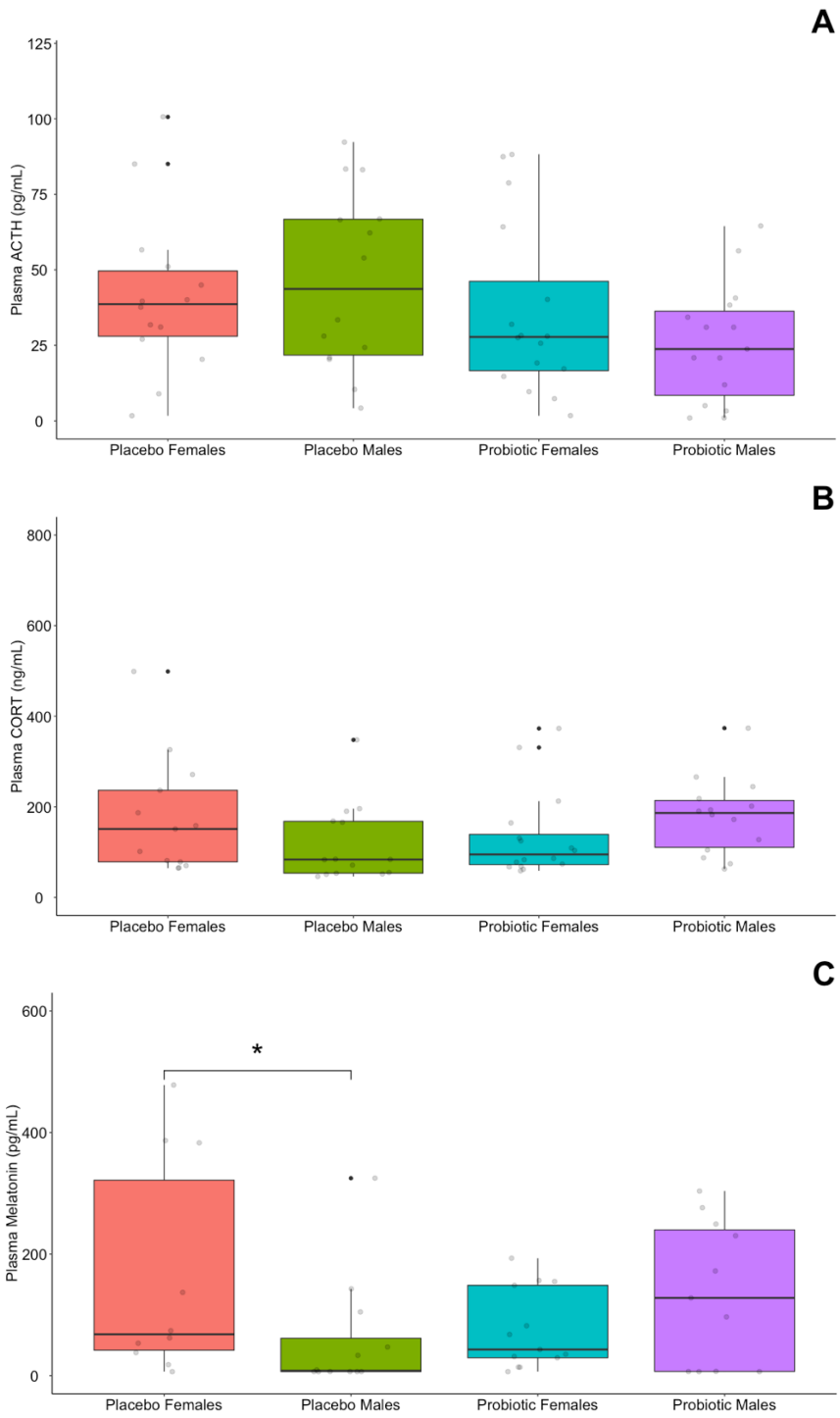

Figure 6 The concentrations of stress hormones in offspring plasma samples for: A) adrenocorticotropic hormone (ACTH) in pg/mL, B) corticosterone (CORT) in ng/mL, and C) melatonin in pg/mL. The median is represented by the thick, black horizontal line, the whiskers represent 1.5 times the interquartile range (1.5 x IQR). The individual transparent dots represent each offspring's raw data for hormone concentration. The solid black dots indicate outliers.

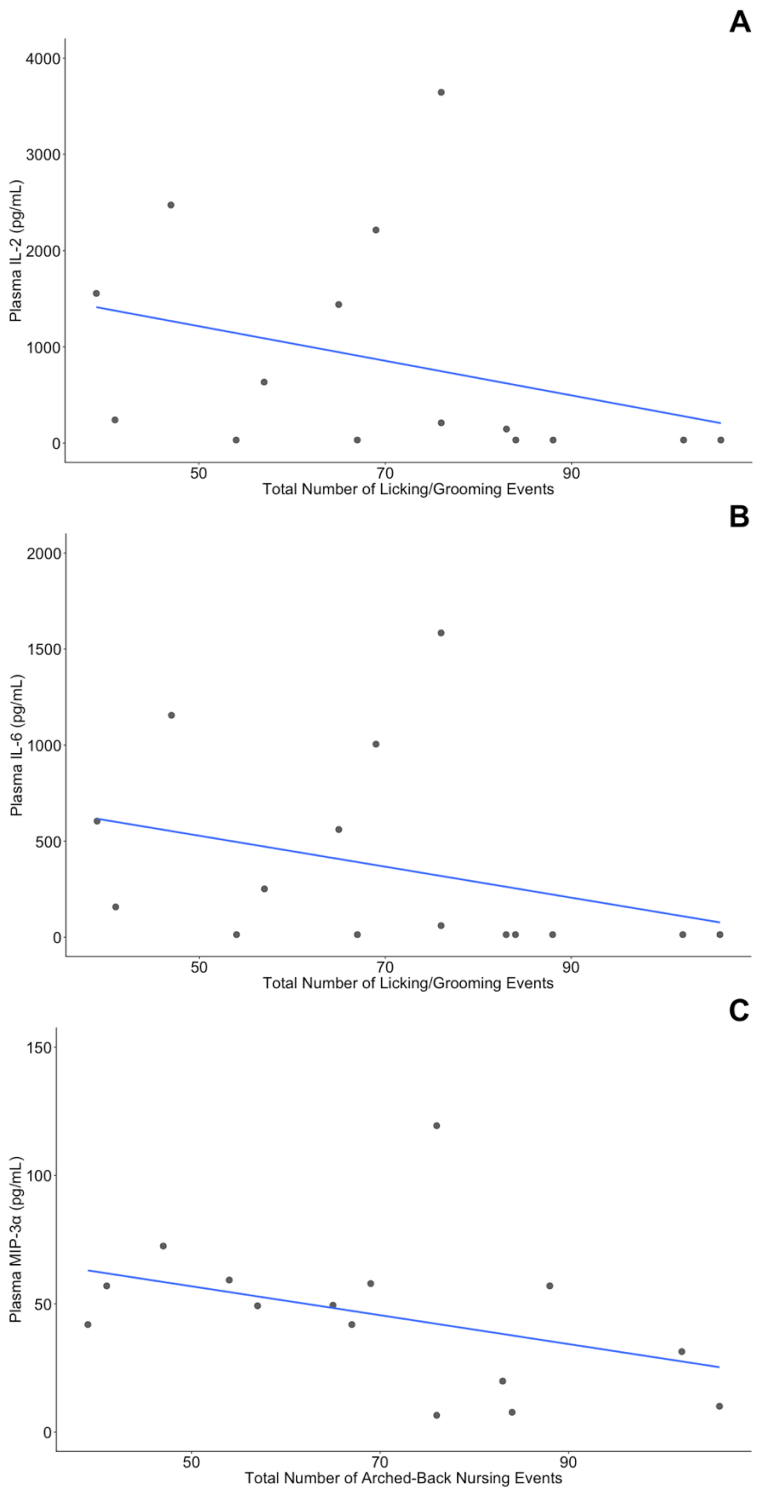

Figure 7 Spearman's correlations represented as scatterplots for A) plasma IL-2 in pg/mL and the total number of licking/grooming events, B) plasma IL-6 in pg/mL and the total number of licking/grooming events, and C) plasma MIP-3a in pg/mL and the total number of arched-back nursing events.

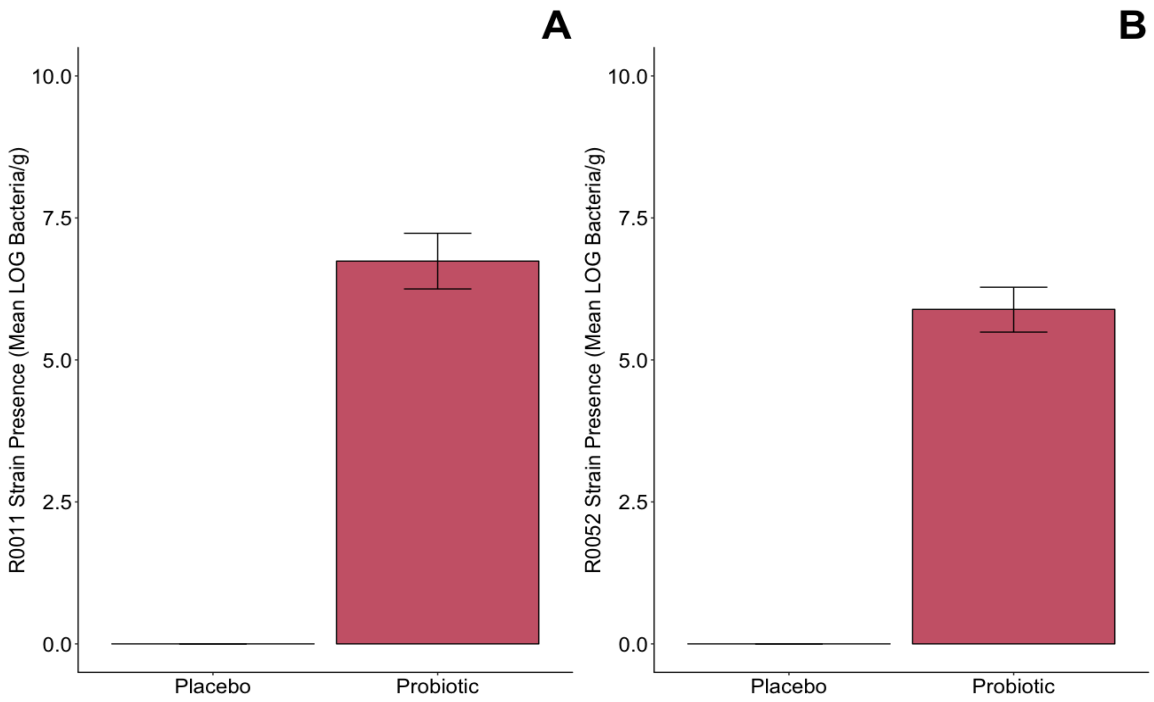

Figure 8 The mean LOG bacteria counts per gram of caecum sample from dams given either placebo or probiotic treatment, with error bars representing standard error for, A) Lactobacillus rhamnosus R0011 and, B) Lactobacillus helveticus R0052.