

IMMUNE CHALLENGE DURING PUBERTY: ROLE OF THE GUT MICROBIOTA AND
NEUROBEHAVIOURAL OUTCOMES

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Abstract

Puberty is a critical period of development characterized by rapid physiological changes and significant brain reorganizing and remodeling. These rapid changes render the developing brain particularly vulnerable to stress and immune challenge. In mice, exposure to an immune challenge (lipopolysaccharide; LPS) during puberty causes enduring effects on stress reactivity, cognitive functioning, and depression- and anxiety-like behaviors later in life. However, the mechanisms underlying these effects are unknown. The gut microbiome can profoundly influence the immune system. There is also close bidirectional communication between the gut microbiome and the central nervous system (CNS) through neural, endocrine and immune signaling pathways, which can alter brain chemistry and emotional behaviour. Thus, we hypothesized that altering microbial composition during puberty could mitigate acute immune responses and prevent enduring outcomes later in life.

The current thesis examined the effect of gut manipulation with probiotics during puberty on LPS-induced immune responses and enduring anxiety- and depression-like behaviours, and stress-reactivity in adulthood, in male and female CD1 mice (Article 1). Next, we examined age and sex differences in gut microbial composition before and after exposure to an immune challenge. We also examined the effects of consuming a single strain probiotic bacterium (*Lactobacillus Reuteri*) during puberty on the immune response and the long-term changes in memory, anxiety-like behavior, and stress reactivity in adulthood (Article 2). Lastly, we examined how microbial colonization between pubertal and adult mice can alter acute peripheral and central inflammatory responses to LPS (Article 3). The current dissertation has addressed sex-specific vulnerabilities to an immune challenge during pubertal development and the moderating influence of the gut microbiome. These studies have demonstrated that manipulating the gut microbiome during puberty can mitigate acute immune responses and prevent enduring neurobehavioural outcomes later in life.

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Conceptual and Theoretical Background

Pubertal Maturation & Onset of Stress-Related Mental Illnesses

Puberty is a critical period of development during which sexual maturity is attained (Sisk & Foster, 2004). Adolescence refers to the time between the beginning of sexual maturation (i.e. onset of puberty) and adulthood and includes physical, psychological and emotional development. Puberty and adolescence are both periods of significant physiological and neurological development (Levitt, 2003; Schulz et al., 2009). Pubertal transition from a non-reproductive to a reproductive state is initiated by the hypothalamic-pituitary-gonadal (HPG) axis which begins with increasing frequency and amplitude of gonadotropin-releasing-hormone (GnRH) secretion. GnRH neurons from the hypothalamus begin to fire in a synchronized fashion, resulting in intermittent pulses of GnRH release (Plant and Barker-Gibb, 2004). Kisspeptin, a neuropeptide and processed product of the *kiss-1* gene, directly stimulates GnRH release by binding to its G protein-coupled receptor (GPR54) located on GnRH neurons (Kauffman et al., 2007) and modulates steroid feedback from the HPG axis (Navarro et al., 2011). The GnRH pulsatile release initiates the production of luteinizing hormone (LH) and follicle-stimulation hormone (FSH) from the anterior pituitary gland which stimulates the gonads to produce gonadal steroid hormones. In addition to kisspeptin, a variety of signals influence GnRH pulses including metabolic (i.e., body fat, leptin), photoperiodic (i.e. melatonin) and psychosocial (i.e. social cues, stress) signals (Ellison et al., 2012; Sisk and Foster, 2004).

The HPG axis primarily controls the secretion of estradiol and progesterone from the ovaries in females and androgens from the testes in males. During the pubertal transition there is a significant rise in circulating gonadal hormones, like estradiol, progesterone, and testosterone. These hormones have far-reaching anatomical, physiological, and behavioral effects, and

stressors experienced during puberty can alter the brain's response to these gonadal hormones (Ismail et al., 2011; Olesen, Ismail, Merchasin & Blaustein, 2011), which will be discussed.

A rise in gonadal hormones during puberty induces several physiological changes including rapid physical growth and the development of secondary sex characteristics (e.g., breast budding in girls and testicular enlargement and growth of chest hair in boys) and the onset of reproductive competence (e.g., menarche and ovulatory menstrual cycles in girls and semenarche and spermatarche in boys) (Dahl & Gunnar, 2009; Patton & Viner, 2007; Tanner, 1962). This period of maturation in humans begins around age 10-11 for girls and is fully completed by age 15-16 (Marshall & Tanner, 1969) whereas for boys, puberty begins slightly later at age 11-12 and males can have ongoing physical changes up until age 16-17 (Tanner, 1962). In mice, the timing of puberty is variable depending on the housing environment and mouse strain, but it is estimated to begin around post-natal day 30 in inbred C57B1/6 and outbred CD1 female mice housed in single sex rooms (Ismail & Blaustein, unpublished observations) and post-natal day 35 in rats (Ojeda and Urbanski, 1994; Ojeda et al., 1976). In mice, the pubertal period can last several weeks when housed in the absence of males (Vandenbergh, 1967; Vandenbergh, 1969). Pubertal onset can be identified by an external marker of gonadal activity which is vaginal opening in females (Lohmiller & Sonya, 2006; Ojeda et al., 2003), and the separation of the prepuce from the glans penis (i.e. preputial separation) in males (Korenbrod et al., 1977). In female mice, puberty is considered complete at the onset of the first reproductive (i.e. estrous) cycle (Vandenbergh, 1967; Vandenbergh, 1969). Outbred CD1 mice are more genetically diverse, compared to inbred mice, and this variation is more reflective of genetic diversity observed in human populations (Aldinger et al., 2009).

During this critical period of maturation, the brain undergoes significant reorganizing and

remodeling, which are primarily driven by gonadal steroid hormones (Levitt, 2003; Sisk & Foster, 2004, Sisk & Zehr, 2005). There is substantial structural and functional remodeling of the central nervous system (CNS) throughout adolescent development (Gogtay, Giedd, Lusk, et al., 2004) including increases in white matter in areas responsible for refined skills in complex attention and language (Paus et al. 1999; Bava et al., 2010), and reductions in cortical gray matter density in the dorsal parietal and frontal regions (Sowell et al., 1996). The trajectory of white matter growth and gray matter volume, particularly in the limbic and cortical regions differs depending on sex (De Bellis et al., 2001; Paus et al., 2001). Adolescent males have higher white matter volume compared to females (Perrin, Herve, Leonard et al., 2008), while females show higher grey-matter density in a number of cortical regions (Paus et al., 2010). The greater white matter volume in pubertal males is associated with increasing testosterone levels (Perrin, Herve, Leonard et al., 2008). The influence on gonadal hormones on brain structure in females remains unclear. Mechanisms affecting the neural circuitry during this stage of development include neurogenesis, myelination, apoptosis, growth of axonal projections and axon sprouting, dendritic elaboration and retraction, synaptogenesis, and synapse elimination (Sisk & Foster, 2004). There are also significant increases in hippocampus and amygdala volume during early pubertal stages (Giedd, et al., 1999; Meyer, Ferres-Torres & Mas, 1978; Romeo & Sisk, 2001). This period of rapid maturation has long-term effects on neurological changes and neural functioning into adulthood (Levitt, 2003; Sisk & Foster, 2004). Puberty can therefore be described as a highly programmed and biologically driven period of development that shapes the CNS.

The incidence of psychiatric mental illnesses, including anxiety, mood disorders, eating disorders, personality disorders, psychosis and substance abuse, increase substantially during

puberty and adolescence in humans (Ge et al., 2001; Kessler et al., 2005; Newman et al., 1996; Silverman et al., 1996). Most adult cases of major depression emerge during adolescence, with the peak age of 14 years for these mental disorders (Kessler et al., 2005; Paus et al., 2008). Sex differences also emerge following puberty in the prevalence of various mental illnesses.

Epidemiological studies show that stress-related mental illnesses often onset during puberty with a higher prevalence rate in females than in males at about a 2:1 ratio (Angold and Costello, 2006). The higher prevalence in females has been directly correlated to pubertal status (tanner stage III), which is a better predictor of sex difference in prevalence than chronological age (Hayward & Sanborn, 2002; Patton et al., 1996). This could be related to sexual dimorphisms in certain regions of the pubertal brain. Brain volume and neural density develop differently during puberty between males and females. Hippocampal volume is greater in girls than in boys, while amygdala volume is greater in boys (Giedd et al., 1997). Steroid hormones play a significant role in brain organization and morphological changes during specific developmental windows (Sisk and Zehr, 2005), including the pubertal period. However, prior to and after these specific developmental windows, sex hormones appear to have no effect on these CNS-level changes (Phoenix et al., 1959). Factors contributing to the emergence of mental illness are complex and unclear. It is theorized that puberty and adolescence is a time of heightened vulnerability to biological and environmental factors including stress and inflammation. Moreover, exposure to stress and inflammation during this period of rapid neurodevelopment alters neurobehavioral processes resulting in lasting effects on adult brain and behaviour (Andersen, 2003; Holder & Blaustein, 2014).

The Stress Response and Puberty

The physiological response to stress is controlled by the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system. The experience of a threat causes sympathetic nerves and the adrenal medulla to release catecholamines, which induce a rapid physical response such as increased heart rate, pupil dilation and decreased gut motility (Kvetnansky et al., 2009). In comparison, the activation of the HPA axis, a major neuroendocrine system, is a slower and often longer response to the experience of a stressor, which responds with the release of glucocorticoids (primarily cortisol in humans and corticosterone in rats and mice). Activation of the HPA axis is primarily controlled by the amygdala and medial prefrontal cortex (mPFC). Anatomical connections between the amygdala, mPFC, hippocampus and hypothalamus facilitate the HPA axis response. The HPA axis is characterized as a circuit in which the paraventricular nucleus (PVN) of the hypothalamus releases corticotropin releasing factor (CRF) which binds to CRF receptors on the anterior pituitary gland. This leads to the release of adrenocorticotrophic hormone (ACTH), which binds to receptors on the adrenal cortex and stimulates release of glucocorticoid hormone into the blood. The glucocorticoid hormone can be released for several hours following a stressor. The negative feedback loop begins when the blood concentration of the glucocorticoid hormone is achieved, after the stressor has terminated, which will signal to the hypothalamus and pituitary to inhibit further secretion. Projections from the hippocampus and infralimbic mPFC also facilitate the negative feedback loop (Ulrich-Lai and Herman, 2009). The HPA axis plays an essential role in the regulation of homeostatic systems including metabolic (Brandi et al., 1993; Rizza et al., 1982; Vitaliano et al., 2002), cardiovascular (Sapolsky and Share, 1994; Whitworth et al., 1995), immune (Straub et al., 2011), reproductive (Besedovsky et al., 2008) and the central nervous (Pariante and Miller, 2001; Tafet and Bernardini, 2003).

There are age and sex differences stress reactivity and responsiveness of the HPA axis. In both inbred and outbred strains of mice, prepubertal males show greater stress-induced hormonal responsiveness compared to their adult counterparts, as shown through elevated and a more prolonged corticosterone (CORT) responses. Inbred female mice show similar stress-induced hormonal responses, independent of age (Romeo et al., 2013). Yet, adult females belonging to the outbred Swiss-Webster strain display heightened CORT response compared to prepubertal females (Romeo et al., 2013). Adult female mice typically demonstrate greater hormonal reactivity to stress compared to adult male mice (Harizi et al., 2007; Goel & Bale, 2008). Age differences in the stress response have also been observed in rats. For example, following exposure to various acute stressors (e.g. ether, foot shock, restraint stress), prepubertal male rats display elevated and more prolonged concentrations of corticosterone and adrenocorticotrophic hormone levels (Romeo, 2010; Romeo, Foilb et al., 2011; Romeo, Kaplowitz, & Ho, 2013; Romeo, Lee, Chua, McPherson, & McEwen, 2004; Romeo et al., 2004a, 2005) and greater and more prolonged activation of early gene expression (i.e. Fos and Egr-1) in the PVN (Viau et al., 2005). Moreover, habituated stress responses are observed in adult rats following repeat stress exposure (Girotti et al., 2006; Harris et al., 2004; Helmreich et al., 1997; Magarinos and McEwen, 1995; Marti and Armario, 1997) but male pubertal rats do not experience this habituation. Instead, pubertal rats experience greater peaks of ACTH, CORT and adrenal progesterone accompanied by more rapid return to baseline (Doremus-Fitzwater et al., 2009; Romeo et al., 2006). Both similarities and differences in the stress responses have been noted between species (e.g. mice and rats) suggesting species-specific differences exist in response to acute stress (Romeo et al., 2013).

Age and sex differences in stress responses are likely due to differences in circulating gonadal hormones. In adult male rats, testosterone is shown to reduce the peak and recovery time of the hormonal stress response (Handa et al., 1994; McCormick, Linkroum, Sallinen & Miller, 2002). In contrast, estradiol plays an inhibitory role in the HPA stress response of adult females (McCormick et al., 2002; Young, Altemus, Parkison, & Shastri, 2001). For example, low dose estradiol treatment for 7 days in ovariectomized female rats decreased ACTH, but not CORT response to restraint (Young et al., 2001). Interestingly, gonadal steroid hormones begin to modulate HPA axis reactivity only during puberty, because the stress response in pre-pubertal males and females is not affected by the presence or absence of gonadal hormones (Romeo et al., 2004a; Romeo et al., 2004b). These findings highlighting that gonadal hormones begin to prime adult-like patterns of stress reactivity throughout puberty (Gomez et al., 2004). Age and sex differences in HPA axis reactivity are also observed in the response an immune challenge. In the past few decades, the importance of the immune system on mental illness has received increased consideration.

Immune System and Puberty

The immune system is a multifaceted and complex system responsible for recognition and response to pathogens. Both HPA axis and the immune system mature during puberty and are thought to play a critical role in shaping developmental outcomes (Holder & Blaustein, 2014). Vertebrates have both an innate and an adaptive immune system. The innate immune system responds to pathogens which are evolutionary conserved such as bacterial, viral, fungal or foreign proteins and responds with a variety of mechanisms to destroy invading microorganisms (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2000). The adaptive immune system is primed throughout the lifespan, through B- and T-lymphocytes, which

respond to pathogens with a specialized cellular defense to target and destroy an infective agent (Janeway, 2001). This specialized cellular defense includes the production of cytokines which are peptides involved in cell signaling and modulate immune responses. Cytokines include chemokines, interferons (IFN), interleukins (IL), lymphokines, and tumour necrosis factors (TNF). There is also an important bi-directional communication and feedback between the HPA axis and immune system. More specifically, a number of cytokines, such as IL-1, IL-6, IL-10 and TNF- α can activate the HPA axis and, in turn, the HPA axis modulates the immune response, with high levels of cortisol resulting in a suppression of inflammatory reactions (Besedovsky et al., 1996; Silverman et al., 2005).

Much of our understanding of the immune response and changes throughout the lifespan comes from research studies using lipopolysaccharide (LPS). LPS is a bacterial endotoxin derived from the cell wall of gram-negative bacteria (e.g. *Escherichia coli*) (Raetz & Whitfield, 2002) and has wide range of uses in research models because of its ability to stimulate the innate immune response. When administered in the periphery, LPS binds to toll-like receptor 4 (TLR-4) on immune cells and triggers an intracellular cascade of events that ultimately results in the production and release of pro- and anti-inflammatory cytokines in the blood (de Bont et al., 1998; Beaty et al., 1994). These molecules are either pyrogenic (fever-inducing) or anti-pyretic (fever suppressing) (Aronoff & Neilson, 2001). LPS also increases cyclooxygenase and prostaglandins (Beutler and Poltorak, 2000) and induces HPA axis activation through triggering the release of corticosterone (Suzuki et al., 1986; Sorells & Sapolsky, 2007). Peripheral LPS and peripheral cytokines can cross the blood brain barrier (BBB) and induces neural activation in the brain by binding to TLR4 in the brain and prompt immune responses (Bilbo & Schwarz, 2009; Kentner et al., 2010) through activation of resident microglia immune cells. This response leads

to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). Increased production of NF- κ B as well as cyclooxygenase-2 (COX-2) indicates inflammation and is involved in the transcription and translation of both pro- and anti-inflammatory cytokines within the CNS (Andreaskos et al., 2004; Maes et al., 2007; Vitkovic et al., 2000).

There are significant age and sex differences in the behavioural and physiological responses to an immune challenge between pubertal and adult mice. Recent findings from our laboratory show that adult male mice display more sickness behavior and greater hypothermia following LPS treatment compared to their pubertal counterparts. Furthermore, both male and female adult mice treated with LPS display greater pro-inflammatory cytokines compared to pubertal mice, while pubertal mice display greater anti-inflammatory cytokines in the blood compared to adult counterparts at 10 hours post-injection (Cai et al., 2016). Adult female mice show greater serum corticosterone concentration compared to adult males and pubertal males and females at two hours post-LPS exposure. At this same time point, LPS-treated adult male and female mice also display an increased number of c-Fos immunoreactive cells in a number of forebrain regions compared to saline-injected controls. However, LPS-treated pubertal mice do not display greater c-Fos expression compared to saline-treated controls (Girard-Joyal et al., 2015). Thus, pubertal mice appear to be hypo-responsive to LPS treatment compared to adult mice. Follow-up studies examining central mRNA cytokine expression to LPS treatment show that pubertal males displayed greater IL-1 β , TNF α , and IL-6 mRNA expression in the prefrontal cortex at 2 hours compared to their adult counterparts, whereas adult males generally display greater cytokine expression at 8 hours (Sharma et al., 2018). Peripheral cytokine immune responses measured in blood serum differ from those seen within the brain (Carvey et al., 2009; Sharma et al., 2018). Taken together, inflammatory responses are highly specific to sex and age.

Immune molecules within the CNS and are thought to play a critical role in brain development during critical periods (Boulanger, 2009; Carpentier and Palmer, 2009; Deverman and Patterson, 2009; Garay and McAllister, 2010). For example, microglia (primary immune cells in the brain) are highly involved in the process of synaptic pruning necessary for normal brain development in the neonatal period (Verney et al., 2010; Graeber, 2010; Schafer et al., 2012). In response to damage or infection, microglia adapt and restructure for quicker future responses (Hinwood et al., 2012; Graeber and Streit, 2010; Kettenmann et al., 2011; Ransohoff and Perry, 2009; Streit et al., 1999), which has important implications on brain development. Peri-pubertal female and adult rats have more activated microglia than males of the same age in the hippocampus, parietal cortex and amygdala, suggesting females are more sensitive to immune challenges during puberty/adolescence and early adulthood. Similarly, female CD1 mice display more activated microglia in the pubertal period than in adulthood (Holder and Blaustein, unpublished observations). Thus, neuroinflammatory activity is thought to be especially influential on morphological restructuring during critical periods of brain development and has been proposed as a pathway for which pubertal complex stressors alter adult brain and behaviours (Holder and Blaustein, 2014).

Vulnerability of Puberty to Stress and Immune Challenges

Adolescent exposure to stressful life experiences can contribute to an individual's enduring vulnerability to mental illness later in life. Humans exposed to chronic or extreme stressors and traumatic experiences during adolescence are more likely to develop a variety of mental disorders such as depression, anxiety and post-traumatic stress disorder (PTSD) (Ge et al., 2001; Grant et al., 2003; Grant et al., 2004; Silverman et al., 1996; Turner and Lloyd, 2004). In rodent models, exposure to stress during puberty causes enduring effects on physiological

(e.g. HPA function), behavioural, and altered neurobiological responses to stress into adulthood (Bazak et al., 2009; Green, Barnes & McCormick, 2012; Jacobson-Pick and Richter-Levin, 2010; Laroche et al., 2009). Exposure to a variety of physical stressors such as heat, immobilization, or ether, during pubertal maturation, causes long-term negative effects on reproductive capacity (i.e. the ability to produce offspring) in female mice (Paris, Kelly & Ramaley, 1973). Similarly, exposing mice of both inbred and outbred strains to shipping stress or to an immune challenge, the bacterial endotoxin, lipopolysaccharide (LPS), during puberty, decreases sexual receptivity in adulthood following treatment with estradiol and progesterone compared to mice stressed earlier or later in development (Laroche et al., 2009a,b; Ismail et al., 2011; Ismail & Blaustein, 2013). Moreover, social instability stress during adolescence increases anxiety-like behaviour and decreases social interaction in adult male rats (Green et al., 2012) suggesting stressful experiences during puberty can have profound and long-lasting effects on stress responses in adulthood. Rats exposed to a short stressful experience (i.e. placement on an elevated platform at 28 days of age) display greater anxiety-like behavior and heightened startle responses in adulthood compared to rats only exposed to it in adulthood (Avital & Richter-Levin, 2005). Moreover, social isolation throughout adolescent development in male mice results in reduced basal CORT levels but heightened CORT reactivity to novel stress compared to non-isolated controls (Ros-Simo & Valverde, 2012). The association between adolescent exposure to stress and long-term impacts on psychological dysfunction has also been observed in humans. For example, heightened stress caused by traumatic experiences (e.g. witnessing physical violence) during the adolescent period is associated with greater occurrences of anxiety and/or depressive disorders in adulthood (Turner & Lloyd, 2004).

Pubertal exposure to an immune challenge (LPS) induces long-lasting changes in the brain and behavior through inflammation and neuroinflammation (Holder & Blaustein, 2014). Neuroinflammation has been linked to the etiology of anxiety (Hou and Baldwin, 2012; Jones and Thomsen, 2012) and mood disorders, including depression and bipolar disorder (Capuron and Miller, 2011; Miller 2009). In mice, pubertal exposure to LPS alters reproductive and non-reproductive behaviors like cognitive functioning and responsiveness to gonadal steroid hormones on anxiety-like and depression-like behaviours, in an enduring manner (Ismail et al., 2013; Ismail et al., 2011; Olesen et al., 2011; Kolmogorova et al., 2019). Pubertal female mice treated with LPS at five or six weeks old, but not at three, four, seven, eight, or ten weeks old displayed decreased sexual receptivity in adulthood, compared to saline-injected controls, even though they were appropriately hormone-primed (Laroche et al., 2009a). These findings suggest that pubertal LPS treatment decreases the behavioural responsiveness to estradiol and progesterone. Estradiol is a known enhancer of performance on hippocampal-dependent tasks and facilitates memory formation (Spencer et al., 2008; Luine and Frankfurt, 2012) and reduces anxiety-like and depression-like behaviours (Okada et al., 1997; Rachman et al., 1998; Marcondes et al., 2001; Dalla et al., 2004; Tomihara et al., 2009). In female ovariectomized mice, treatment with estradiol and progesterone in adulthood reduces anxiety-like and depression-like behaviour (Olesen et al., 2011; Ismail et al., 2013). However, exposure to LPS during puberty blocks the effects of estradiol and progesterone to reduce anxiety-like behaviour in adulthood (Olesen et al., 2011). Moreover, female mice treated with LPS during puberty show an increase in depression-like behaviour when treated with estradiol in adulthood (Ismail et al., 2013).

Pubertal LPS treatment also causes enduring effects on cognition. Specifically, females exposed to LPS during puberty become less responsive to the beneficial effects of estradiol and perform worse on tasks including social discrimination, social recognition, object recognition and object placement tasks compared to controls (Ismail & Blaustein, 2013). Taken together, the beneficial effects of estradiol and progesterone on behaviour are not seen in mice treated with LPS during puberty (Ismail et al., 2011; Olesen, Ismail, Merchasin & Blaustein, 2011). More recent findings show that a single injection of LPS during puberty in CD1 mice altered hippocampus-dependent learning and memory processes (Kolmogorova et al., 2019) and increased Parkinson-like behaviour through changes to tyrosine hydroxylase expression in adult males (Girard-Joyal & Ismail, 2017). Overall, pubertal exposure to an immune challenge alters responsiveness to gonadal steroid hormones in anxiety- and depression-like behaviours and induces impairments in cognition in adulthood. The mechanisms underlying these LPS-induced enduring behavioral changes remain unknown.

The Gut Microbiome

One possible mediator influencing enduring effects of an immune challenge could be the gut microbiome, as it is a known regulator of systemic immune responses (Belkaid & Hand, 2014; Rooks & Garrett, 2016; Fung, Olsen, Hsiao, 2017). A growing body of evidence suggests a fundamental role of the gut microbiome on brain and behaviour (Cryan & Dinan, 2012; Sampson & Mazmanian, 2015). However, the role of gut microbiota during pubertal development remains largely uninvestigated. The gastrointestinal (GI) tract is a collection of organs related to digestion and include the esophagus, stomach and intestine. The GI tract contains approximately 1×10^{14} microorganisms (Gill et al., 2006; Qin, Raes, Arumugam et al., 2010). These microorganisms include bacteria, archaea, viruses and eukaryotic microbes that have the potential to influence

our physiology and are commonly referred to as the gut microbiome. Advancements in the identification of bacterial composition in the microbiome has allowed for research to begin to understand the structure and function of the microbiome when in healthy versus disease-specific states (Lozupone et al., 2012; Shreiner, Kao & Young, 2015). In humans, healthy individuals harbor more than 1000 species of bacteria belonging to few known bacterial phyla; Bacteroidetes and Firmicutes being the dominant phyla (Lozupone et al., 2012). These microbes play a vital role in various functions such as nutrient absorption, homeostasis of the intestinal barrier, and immune response. The immune system and gut microbiota maintain a symbiotic relationship which allows for proper defensive responses to invading pathogens (O'Hara and Shanahan, 2006; Belakaid & Hand, 2014). The stability and gut bacterial composition can be altered by a number of factors including bacterial infections, gastrointestinal disorders (e.g. irritable bowel syndrome; IBS), diet, consumption of probiotics and antibiotics, and stress. Changes to stability of the gut results in various harmful effects on health and well-being and gut microbial imbalance plays a large role in a variety of disease states such as IBS, autism, obesity, and multiple sclerosis. Therefore, bacterial communities directly contribute to health outcomes (Cryan & O'Mahony, 2011; Cryan & Dinan, 2012).

The gut flora has been highly studied in relation to physical health; however, research is just beginning to elucidate its impact on the CNS. The connection between the gut microbiome and the CNS is commonly referred to as the gut-brain axis (Cryan & O'Mahony, 2011; Rhee, Pothoulais & Mayer 2009). This axis involves several pathways including endocrine (e.g. cortisol), immune (e.g. cytokines) and neural (e.g. vagus and enteric nervous system) to impact brain biochemistry and emotional behaviour but the extent of these communications is not fully understood (Cryan & Dinan, 2012; Gareau, Wine, Rodrigues, et al., 2011). The vagus nerve

(cranial nerve X) consists of both afferent and efferent fibers that provide autonomic regulation of many organs including those of the gastrointestinal tract. The autonomic system, with the sympathetic and parasympathetic limbs, uses both afferent signals, beginning from the intestinal lumen and conveys signals to the CNS through enteric, spinal and vagal pathways and efferent signals from CNS to the wall of the intestine. The enteric nervous system comprises sensory nerves that contribute to gut reflexes and provides information to the brain while the effector limb integrates physiological information about the gut and also modulates immune activity (Fung, Olsen, Hsiao, 2017). Gut-brain signaling is therefore, a bi-directional signaling pathway affecting both cognitive functioning and gut inflammation (Cryan & Dinan, 2012; Cryan & O'Mahony, 2011; Gareau, Wine, Rodrigues, et al., 2011).

The gut microbiome is suggested to contribute to normal brain development (Borre et al., 2014; Jasarevic et al., 2016). Exposure to prenatal stress or microbial pathogen infections during prenatal development in humans is associated with increased susceptibility to neurodevelopmental disorders such as autism and schizophrenia (Mittal et al., 2008; Bale et al., 2010). In rodent models, exposure to microbial pathogens at similar time points in prenatal development result in enduring behavioural abnormalities such as increases in anxiety-like behaviour and impairments in cognition (Goehler et al., 2008; Bilbo et al., 2005). Moreover, abnormal gut microbial composition during critical periods in neurodevelopment is believed to contribute to a variety of neurological disorders. For example, germ-free (GF) mice (i.e. mice lacking gut microorganisms) display higher numbers of immature microglia in the gray and white matters of the cortex, hippocampus, corpus callosum, and other regions compared to conventionally colonized controls (Erny et al., 2015). An interesting phenomenon is the potential role of the gut in Autism Spectrum Disorder (ASD) (de Theije et al., 2011), a disorder defined by

abnormal brain development affecting social and communication skills. Gastrointestinal symptoms including abdominal pain, constipation, diarrhea and bloating are often reported in children with autism and these children are often exposed to antibiotic therapy (Finegold et al., 200; Parracho et al, 2005). Analysis of faecal flora of children with ASD reveal greater toxin-producing bacteria, including neurological toxins (Hatheway, 1990; Parracho et al., 2005; Finegold et al., 2010; Song et al., 2004). Treatment with specific antibiotics that target gram positive anaerobes is suggested to improve autistic symptoms (Sandler et al., 2000). This finding has led to the proposition that gastrointestinal disturbances and microbiota that colonize the GI tract during critical periods in development exert a strong and enduring influence on the brain (Heijtz et al., 2011). However, very little is known about the influence of gut microbiota on the neurodevelopment during puberty and adolescence.

Microbiota role in stress and behaviour disorders. The gut microbiome is becoming increasingly recognized for its importance in stress-related psychiatric disorders, like anxiety. Perhaps some of the best available evidence for the influence of gut microbiota on stress-related disorders comes from the gastrointestinal disorder known as irritable bowel syndrome (IBS). There is high comorbidity between IBS and anxiety (Codling et al., 2010; Reber, 2012) and heightened stress often leads to gut inflammation in these individuals (Logan & Katzman, 2005; Mayer, 2011). The gut microbiome can also modulate the stress response and HPA axis reactivity (Bailey et al., 2011; Crumeyrolle-Arias et al. 2014; Gareau et al., 2011) and intestinal microbiota play an important role in the development of the HPA axis (Sudo et al., 2004). For example, exaggerated ACTH and corticosterone responses to mild restraint stress are observed in GF mice compared to mice raised with normal microbiota composition. These same mice also show reduced brain derived neurotrophic factor (BDNF) expression in the cortex and

hippocampus. Colonizing GF mice with commensal microbiota after birth can partially reverse altered HPA stress reactivity but only if exposure to these microbes occurs during this stage of development and not later (Sudo et al., 2004). Exposure to stress early in life also alters the composition of the gut microbiota, increases visceral sensitivity and changes CNS neurochemistry which points strongly to an important gut-brain interaction on programming future stress reactivity (O'Mahony et al., 2009). These results suggest that microbes have very specific priming effects on brain neurochemistry and stress responses during development. In another study, colonization of GF mice with microbiota from another mouse strain caused previously GF mice to exhibit a similar behavioural profile and display reduced anxiety-like behaviour (Bercik et al., 2011). Therefore, gut microbes contribute to HPA axis responsiveness and are critical to the development of an appropriate stress response later in life.

Gut microbiota composition is also suggested to directly affect an individual's susceptibility to anxiety and depression. In a study by Bercik and colleagues (2010), male mice infected with a bacterial parasite displayed increased anxiety-like behaviour and changes in the CNS, including decreases in hippocampal levels of BDNF mRNA, which have been associated with anxiety-like and depression-like behaviour (Martinowich, Manji & Lu, 2007). Stimulation of the vagal nerve (vagal nerve stimulation therapy; VNS therapy) in humans causes significant improvements in mood (Elger et al., 2000; Nemeroff et al., 2006; Schmidt, 2001) and the vagus nerve is suggested as the main signaling pathway by which the gut microbiome alters behaviour and stress. However, vagotomy prior to an infection with a bacterial parasite in mice does not prevent increased anxiety-like behaviour (Bercik et al., 2010). These studies provide important insight into a complex communication between the gut and CNS through multiple pathways contributing to mood and anxiety. Perhaps some of the strongest evidence for this has been conducted with

the use of probiotic agents, which will be discussed.

Microbiota role with cognition, memory and learning. Alteration to gut microbial composition affects other cognitive processes such as learning and memory (Li et al., 2009; Gareau et al., 2011; Cryan & Dinan, 2012). Gareau and colleagues (2011) determined that 5-6-week-old GF Swiss-Webster mice display deficits in non-spatial, working memory and reference memory tasks (types of short-term memory that are essential to the long-term storage of information). Moreover, in female specific pathogen-free (SPF; free of particular pathogens) C57BL/6 mice, infection with *Citrobacter Rodentium* (i.e. a bacterial infection known to induce colitis) caused significant changes to gut microbiota composition and facilitated stress-induced memory dysfunction. These mice displayed poorer performance on behaviour tests of working memory, reference memory and spatial memory (novel object test and T-maze) following exposure to stress (water avoidance test) compared to sham-infected female controls (Gareau et al., 2011). The composition of the gut microbiota is further suggested to directly improve memory and learning. Working and reference memories (assessed using the hole-board apparatus test), were significantly improved in male mice fed a diet that increased overall gut bacterial diversity compared to controls (Li et al, 2009).

A link between memory and gut dysfunction has also been observed in humans. Bacterial and a variety of viral infections (e.g. herpes simplex virus) have been associated with an increased risk of Alzheimer's disease (AD; Huang et al., 2014; Karim et al., 2014; Lurain et al., 2013; Hammond et al., 2010). This research postulates an important microbe-dementia connection; pathogenic microbes can promote chronic inflammatory responses in the CNS, and this could be a key contributor to neurodegeneration (Huang et al., 2014). Chronic inflammation results in changes to amyloid- β peptides, causing amyloidosis (abnormal deposits and buildup of

the protein, amyloid), a hallmark of AD (Glass et al., 2010). While the pathogenesis of dementia and other memory impairments remains poorly understood, this research proposes that the maintenance of healthy gut flora could be a potential avenue for preventing learning and memory deficits and related diseases.

Probiotics

The World Health Organization (2001) defines probiotics as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host". Gut microbiome composition can be modified directly through ingestion of probiotics. The health benefits of probiotics are dependent on the strain of bacteria, dosage, disorder that is being treated, and frequency of consumption (Sherman, Ossa, Johnson-Henry, 2009). Probiotic research has been very successful at demonstrating the connection between the gut and the CNS (Gareau et al., 2007; Mckernan et al., 2010; Hemarajata et al., 2013; D'Mello et al., 2015). Probiotics are emerging as a potential therapeutic agent for stress-related gastrointestinal problems. Thus far, probiotics have shown benefits for the treatment of some IBS symptoms (O'Mahony et al., 2005; Kajander et al., 2008). Patients with IBS have shown some clinical evidence for the role of probiotics on reductions in physical and psychological symptoms such as anxiety and stress with improvements in mood (Logan & Katzman, 2005; Pinto-Sanchez et al., 2017).

Probiotic intervention reduced anxiety, depressive mood, stress responses and improved cognition in a number of studies (Messaoudi et al., 2011; Bravo et al., 2011; Arseneault-Breard et al., 2012; D'Mello et al., 2015). A study by Bravo, Forsythe, Chew et al. (2011) reported that male mice treated with the probiotic *Lactobacillus rhamnosus* (JB-1) chronically over 28 days resulted in lower levels of plasma corticosterone concentrations at 30-minutes following

exposure to forced swim test and reduced anxiety-like behaviour in the elevated plus maze. Moreover, these mice displayed central alterations of GABA_{B1b} mRNA with increased expression in cortical regions and decreased expression in the amygdala, hippocampus and locus coeruleus. GABA is the main inhibitory neurotransmitter and alterations to GABA receptor expression are implicated in the pathogenesis of anxiety and depression (Cryan & Kaupmann, 2005). In a preclinical study in rats, the probiotic *Bifidobacteria infantis* increased plasma tryptophan and kynurenine levels (Desbonnet et al., 2008), which are an essential amino acid and metabolite of interest in the relationship to psychiatric disorders and neurodegenerative conditions (O'Mahony et al., 2015). *Bifidobacteria infantis* also alleviated visceral hypersensitivity (i.e. abdominal pain) in rats (McKernan et al., 2010), which has important implications for human populations with IBS (Clarke et al., 2012). In a human clinical study, healthy volunteers given *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 or placebo in a double-blind, randomized parallel group study for 30 days revealed that probiotics had beneficial psychological effects which was assessed with a variety of measures including; Hopkins Symptom Checklist (HSCL-90), the Perceived Stress Scale, the Coping Checklist (CCL), the Hospital Anxiety and Depression Scale (HADS), and 24 h urinary free cortisol (UFC) (Messaoudi, Lalonde, Violle et al., 2011). This same probiotic combination also reversed the depression-related behavioural effects observed post-myocardial infarction and reduced apoptosis tendency in the limbic system in rats (Arseneault-Breard et al., 2012). Species of *Lactobacillus*, such as *Lactobacillus reuteri* (*L.reuteri*), maintain intestinal barrier function (Ahrne & Hagslatt, 2011), which may protect against vulnerability to pathogens. Exposure to stress is known to enhance susceptibility to infections. However, consumption of *L.reuteri* attenuates stress-enhanced severity of infection (Mackos et al., 2013). Taken together, probiotics

are suggested to have protective effects against cognitive decline and produce beneficial effects on stress, anxiety and mood. Given the beneficial effects species within the *Lactobacillus* genera, we hypothesized probiotics containing species of *Lactobacillus* would produce positive and protective effects during pubertal development.

Current Study and Objectives

The following studies examined the effects of consumption of a probiotic cocktail (kefir) during puberty against LPS-induced immune responses and enduring depression- and anxiety-like behaviors in adulthood (Article 1), age and sex differences in the gut microbiome as a result of LPS treatment and the mitigating effects of *Lactobacillus reuteri*, against LPS-induced long-term effects on anxiety, cognition and stress responses (Article 2) and colonization of the gut microbiota between pubertal and adult mice on acute immune responses (Article 3). It was hypothesized that LPS would cause acute and enduring changes to gut bacterial diversity, and ingestion of probiotics throughout pubertal development would serve as a protective factor against the negative consequences of LPS on both acute sickness responses and enduring changes on behaviour in adulthood. It was also expected that transferring gut microbiota between adult and pubertal mice would alter age-specific immune responses. More specifically, adult mice were expected to show a similar behavioural and inflammatory profile to that of pubertal mice following exposure to LPS (i.e. greater increases in peripheral anti-inflammatory cytokines), as a result of greater gut bacterial diversity. Overall, the goal of these experiments was to further our understanding of the connections between the brain, immune system and the gut microbiome during pubertal development.

Article 1

Probiotic consumption during puberty mitigates LPS-induced immune responses and protects against stress-induced depression- and anxiety-like behaviors in adulthood in a sex-specific manner.

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Abstract

Puberty/adolescence is a significant period of development and a time with a high emergence of psychiatric disorders. During this period, there is increased neuroplasticity and heightened vulnerability to stress and inflammation. The gut microbiome regulates stress and inflammatory responses and can alter brain chemistry and behaviour. However, the role of the gut microbiota during pubertal development remains largely uninvestigated. The current study examined gut manipulation with probiotics during puberty in CD1 mice on lipopolysaccharide (LPS)-induced immune responses and enduring effects on anxiety- and depression-like behaviours and stress-reactivity in adulthood. Probiotics reduced LPS-induced sickness behaviour at 12 hours in females and at 48 hours following LPS treatment in males. Probiotics also reduced LPS-induced changes in body weight at 48 hours post-treatment in females. Probiotic treatment also prevented LPS-induced increases in pro- and anti-inflammatory peripheral cytokines at 8 hours following LPS treatment, reduced central cytokine mRNA expression in the hypothalamus, hippocampus and PFC, and prevented LPS-induced changes to in the gut microbiota. A single exposure to LPS during puberty resulted in enduring depression-like behaviour in female mice, and anxiety-like behaviour in male mice in adulthood. However, pubertal exposure to probiotics prevented enduring LPS-induced depression-like behaviour in females and anxiety-like behaviors in males. Moreover, probiotics altered toll-like receptor-4 activity in the paraventricular nucleus of the hypothalamus (PVN) in males in response to a novel stressor in adulthood. Our results suggest that the gut microbiome plays an important role in pubertal neurodevelopment. These findings indicate that exposure to probiotics during puberty mitigates inflammation and decreases stress-induced vulnerabilities to emotional behaviours later in life, in a sex-specific manner.

Keywords: Adolescence, Development, Inflammation, Gut-Brain Axis, Cytokines, Sex, Immune system, Lipopolysaccharide, Mice

1. Introduction

Depression and anxiety disorders are the leading causes of disability and overall burden of disease worldwide; with depression affecting a staggering 320 million and anxiety affecting 260 million people globally (World Health Organization, 2017; Friedrich, 2017). Despite decades of research on these topics, the biological mechanisms that underlie depression and anxiety remain complex and unclear. The incidence of psychiatric mental illnesses, including depression and anxiety increases substantially during adolescence, with a 2:1 higher prevalence rate in girls than in boys (Newman et al., 1996; Ge et al., 2001; Kessler et al., 2005; Paus et al., 2008). Moreover, exposure to stressful life experiences during adolescent development further increases the probability of developing mental illness later in life (Ge et al., 2001; Grant et al., 2003; Grant et al., 2004; Turner & Lloyd, 2004). There is significant brain reorganizing and remodeling occurring during puberty (Levitt, 2003; Sisk & Foster, 2004, Sisk & Zehr, 2005). These changes are thought to render the brain particularly vulnerable to stress and inflammation and produce long-lasting effects on neurochemical and behavioural outcomes into adulthood (Levitt, 2003; Andersen, 2003; Sisk & Foster, 2004; Holder & Blaustein, 2014).

A growing body of research suggests a central role of the immune system and neuroinflammation in the etiology of mental disorders like anxiety and major depression (Capuron and Miller, 2011; Miller et al., 2009; Herman & Pasinetti, 2018; Poletti et al., 2017). Pubertal exposure to a bacterial endotoxin (lipopolysaccharide; LPS) causes enduring changes in cognitive functioning (Ismail & Blaustein, 2013; Kolmogorova et al., 2019), depression-like (Ismail, Kumlin & Blaustein, 2013) and anxiety-like (Olesen et al., 2011) behaviors in adult female mice. Moreover, LPS treatment causes a long-term reduction in neurogenesis, decreases synaptic connections and dendritic length in the hippocampus (Valero et al., 2014), and can

predispose the developing brain to neurodegenerative conditions, like Parkinson-like behaviour (Girard-Joyal & Ismail, 2017). These effects are limited to LPS exposure at 6 weeks of age as LPS treatment at younger or older ages fail to induce enduring behavioral alterations (Ismail et al., 2011; Ismail et al., 2013; Laroche et al., 2009; Olesen et al., 2011). The mechanisms influencing these long-term negative consequences remain unclear.

The gut microbiome is a complex and dynamic system comprised of trillions of microorganisms (Gill et al., 2006; Qin et al., 2010). These microbes play a central role in the regulation and maturation of the immune system and overall health (Fung et al., 2017; Belkaid & Hand, 2014; Honda & Littman, 2016). Mental health and neurological development appear to be strongly influenced by the microbial composition of the gut microbiome (Cryan & Dinan, 2012; Sampson & Mazmanian, 2015; Tilisch, 2014). Moreover, chronic gastrointestinal infections, which alter the gut, result in increased anxiety-like behaviour, production of pro-inflammatory cytokines, and neuroinflammation (Bercik et al., 2010). The relationship between the gut and the brain is bi-directional; environmental stressors, psychological factors and neuroinflammation can trigger visceral hypersensitivity, a hallmark of irritable bowel syndrome (Coutinho et al., 2002; Chung et al., 2007). In turn, alterations in the gut through consumption of a probiotic (*Lactobacillus rhamnosus*) alters GABA_A and GABA_B receptor mRNA expression in several brain regions. For example, GABA_B receptors were reduced in the amygdala and hippocampus while GABA_A were reduced in the cingulate and prelimbic cortices. Changes in the expression of these receptors has been associated with anxiety and depression-like behaviours in mice (Bravo et al., 2011). Probiotics (live microorganisms) can also have beneficial effects on the stress response and mediate antidepressant effects by modulating inflammatory responses (Park et al., 2018; Baharav et al., 2004; Rao et al., 2009; Messaoudi et al., 2011). However, the role of gut

microbiota during puberty on stress- and immune-induced neurochemical and behavioural outcomes remains uninvestigated.

We believe that the relationship between inflammation, the gut microbiota, and the CNS is particularly crucial during pubertal development. We aimed to determine whether gut manipulation with probiotics can alter acute and enduring consequences of an immune challenge at six weeks old, during the stress-sensitive pubertal period. While most studies to date have examined the short-term cognitive and behavioural effects of probiotics through administration of one or two strains of bacteria (Messaoudi et al., 2011; Bravo et al., 2011; Chung et al., 2014), we selected a probiotic cocktail (i.e. kefir) because it is a commercially available, widely consumed product suggested to provide a host of health benefits (Bourrie et al., 2016), and offers clinical relevance to our results. We tested the hypothesis that probiotic treatment would mitigate LPS-induced immune responses, and enduring effects on anxiety- and depression-like behaviours in adulthood. The primary receptor responsible for LPS-induced immune activation is toll-like receptor 4 (TLR4) (de Bont et al., 1998; Beaty et al., 1994). Therefore, we also investigated the long-term impacts of LPS and probiotic treatments on stress reactivity and TLR4 expression, in the paraventricular nucleus of the hypothalamus (PVN), following a novel stress exposure in adulthood. We hypothesized that pubertal LPS treatment would induce greater stress reactivity and TLR4 expression following exposure to a heterotypic stressor in adulthood. We also expected that probiotic treatment would mitigate these enduring effects.

2. Methods

2.1 Animal Housing

80 male and 80 female CD-1 mice were shipped from Charles River Laboratories (St-Constant, Québec) at 3 weeks of age. Mice were housed in pairs in Polycarbonate cages in either an all-

male or all-female colony room with *ad libitum* access to food and water. Housing rooms were kept at constant temperature ($24 \pm 2^\circ\text{C}$) with a reversed light:dark cycle (14:10; lights off at 10:00am). All procedures were approved by the Animal Care Committee of the University of Ottawa.

2.1.1. Probiotics

Mice received probiotics treatment from 5-7 weeks of age during their pubertal period of development. Lyo San Inc. (Lachute, QC) supplied a lyophilized kefir culture with a lactic acid bacteria concentration of 3.0×10^9 CFU/g which was stored at -20°C . Kefir contained active bacterial culture (*L. lactis*, *L. cremoris*, *L. diacetylactis*, *L. acidophilus*), lactic yeasts and skim milk powder. The kefir was prepared by mixing 5g of dry kefir probiotic culture into 1L of skim milk and left in an airtight container to inoculate at room temperature (23°C - 25°C) for 24 hours before being refrigerated for at least 8 hours to stop the reaction. The control group received the same skim milk with no added probiotics. A new batch of kefir was prepared 3 times per week. Bottles were replaced every 24 hours and vortexed regularly to maintain liquid consistency. To ensure consumption of probiotic treatment, mice had *ad libitum* access to kefir or control skim milk, and water was not available during this period. Bottles were weighed daily to ensure consumption. There was no difference between kefir and control consumption.

2.1.2. Lipopolysaccharide (LPS)

Lipopolysaccharide (obtained from Escherichia coli serotype O26:B6; #L3755; Sigma Aldrich Canada, Oakville, ON) was prepared by dissolving lyophilized LPS into 0.9% (w/v) sterile aqueous sodium chloride (saline) solution (Ricca Chemical Company; #R7210000-4A7210-1). All mice were injected intraperitoneally at 6 weeks of age at the end of the light cycle with either 0.9% sterile saline or LPS (1.5mg/kg). This dose of LPS causes mild sickness in mice for up to

48 hours (Ismail, Garas & Blaustein, 2011) and produces enduring impairments in reproductive and non-reproductive behaviors, when administered at 6 weeks of age, during the stress-sensitive pubertal period (Laroche et al., 2009; Ismail, Kumlin, Blaustein, 2013; Valero et al., 2014; Komogorova et al., 2019). Under our particular housing conditions, female CD1 mice housed in single sex rooms display vaginal opening around postnatal day 30 (Holder & Blaustein, 2014) and first estrus is 20 days post-vaginal opening (N. Ismail and J.D. Blaustein, unpublished observations). In order to minimize additional manipulation and handling stress to females in the current experiment, vaginal smears were not collected. While it is difficult to identify preputial separation in male mice, measurements of scrotum width indicate that the scrotum size of 6-week old males have not yet reached maximum adult size (Lamba, Murray & Ismail, unpublished observations), suggesting that our 6 weeks old mice are indeed pubertal mice.

2.1.3. Sickness Monitoring

Sickness monitoring was conducted at 2, 4, 8, 12, 24- and 48-hours following injection.

Assessment of the progression of pathogen-induced sickness behaviors at these time points followed a non-invasive and unbiased approach by two raters blind to experimental conditions (as described in Kolmogorova, Murray, Ismail, 2017). Mice were assessed for symptoms including lethargy (diminished locomotion), huddling (curled body posture), ptosis (drooping eyelids), and piloerection (erection of hairs). These symptoms were evaluated with respect to the number of symptoms displayed at each time point (one symptom = score of 1; two symptoms = score of 2, three symptoms = score of 3; four symptoms = score of 4). Animals were weighed 48 hours following saline or treatment to assess changes in body weight. Body weight was also assessed from the beginning to the end of probiotic treatment, and again in adulthood.

2.1.4. Multiplex Luminex Immunoassay

Forty male and 40 female mice received an intraperitoneal injection of Euthanyl (prepared from Euthansol; Merck Animal Intervet Canada Corp; Kirkland, Quebec) at 8 hours following saline or LPS injection to examine the effects of probiotic treatment on LPS-induced cytokine responses in blood serum and brain and sex-based differences. Trunk blood was collected using capillary tubes. The following day, tubes were centrifuged at 10,000g at 20°C for five minutes in order to separate blood serum, which was then stored at -80°C until analysis. A multiplex Luminex immunoassay was used to measure serum concentrations of the interleukins (IL)-1b, 6, 10, 12(p70), interferon- γ (IFN γ), and tumour necrosis factor α (TNF α). Assay buffer solution was used to dilute the serum by a factor of two. Serial dilutions are then prepared for mouse cytokine standards for concentrations 3.2, 12, 80, 400, 2000 and 10,000 pg/ml. Then, a cytokine bead solution was added to the plate containing the standards, samples, and controls. The plate was covered, placed on a shaker at 2-8°C, and left to incubate for 16 hours. Detection antibodies were added, and the plate was left to incubate on a plate shaker at room temperature for one hour. Streptavidin-phycoerythrin was added to each well and then incubated on a plate shaker at room temperature for 30 min. After washing, the beads were re-suspended in drive fluid and a MAGPIX system was used to measure cytokine concentrations.

2.1.5. Real-time qPCR

Brains were extracted, frozen using liquid nitrogen, and stored at -80°C. Brain samples were dissected to collect the prefrontal cortex (PFC), hippocampus, and hypothalamus, following guidelines from *The Mouse Brain Atlas in Stereotaxic Coordinates* (Franklin and Paxinos, 1997). Messenger RNA (mRNA) was extracted from frozen brain tissue using Isol-RNA lysis Reagent (Cat. No. 2302700, Fisher Scientific). The extracted RNA was then treated with DNase to remove any genomic DNA prior to cDNA synthesis using the QuantiTect Reverse Transcription

kit (Cat. No. 205311, Qiagen). The resulting cDNA aliquots were used to identify relative gene expression, which was measured using the RealMasterMix Fast SYBR kit (Cat. No. 1725201, Bio-Rad) in 10 μ L reactions on a CFX96TOUCH real time PCR machine. All primers were ordered through Integrated DNA Technologies and primer efficiency was determined using the slope of the relation between RNA quantity and cycle thresholds (C_T) using Bio-Rad software technology. After running standard curves, all primer pairs in this experiment achieved reaction efficiencies between 90% and 110%. All primers were diluted to a final concentration of 0.3 μ M for the real-time PCR reaction (Table 1).

β -actin was used as the reference/housekeeping gene for all samples. No significant difference in β -actin mRNA expression was found between experimental groups. For each reaction, the quantitative threshold amplification cycle number (C_q) was determined by taking the average across groups, and the $2^{-\Delta\Delta C_q}$ method was used to calculate the relative gene expression.

2.1.6. DNA Extraction from Fecal Samples & Amplicon Sequencing

To collect fecal samples, mice were removed from their home cage briefly to induce defecation. Fecal samples were collected at five time points; 5 weeks of age (before probiotic treatment), 6 weeks of age (after one week of probiotics and just before LPS injection), 24 hours after LPS injection, 7 weeks of age (at the end of two-week course of probiotic treatment), 10 weeks of age (adulthood). Upon collection, samples were immediately frozen using liquid nitrogen, placed in Eppendorf tubes and stored at -80°C until DNA extraction. All fecal pellets from paired cage-mates were combined, homogenized and analyzed as a single sample. DNA extraction was performed using a PowerSoil DNA Isolation Kit (MO BIO Laboratory Inc.) according to the manufacturer's recommendations. Fecal samples were thawed and homogenized by vortexing for

3 minutes. 1ml aliquots of each sample were centrifuged at 13,200 rpm (16,100 rcf) for 10 minutes at room temperature. The supernatant was discarded, and the remaining pellet was resuspended in 400µl of nuclease-free water. DNA concentration and purity were evaluated by optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at wavelengths of 230, 260 and 280 nm. We tested our samples for purity (260/280) and all samples revealed a reading ratio between 1.6-3.2. Subsequently, extracted cDNA samples were sent to the Integrated Microbiome Resource (IMR) (Dalhousie University, Halifax, Canada) where amplicon sequencing (MiSeq) was performed for the identification of V6 to V8 regions of 16S rRNA bacterial diversity (as described in Comeau, Douglas & Langille, 2017). Bioinformatics was used to classify reads into different operational taxonomic units (OTUs; at 97% identity).

2.1.7. Elevated Plus Maze and Open Field Test

As of 10 weeks of age, mice were subjected to a variety of behaviour tests. Behaviour testing was carried out over two weeks with a 48-hour rest period between each test to avoid carry-over effects. Animals were exposed to the behavior tests in a consistent order, but we varied the testing order between mice such that males were tested first on the first day and females were tested first on the next day. The Elevated Plus Maze (EPM) and Open Field Test (OFT) were carried-out to examine anxiety-like behavior and locomotion. The Elevated Plus Maze (EPM) consisted of four branching arms (each arm is 10 X 50 cm long), two of which were open and two were closed. Mice were placed in the center facing one of the open arms and allowed to explore the maze for 5 minutes. The Ethovision Tracking software was used to measure the amount of time spent in each portion of the maze throughout the 5-minute trial. An increased amount of time spent in the closed arms of the maze is related to greater anxiety (Roy and

Chapillon, 2004; Wall and Messier, 2001). For the OFT, mice were placed in the arena for 5 minutes and locomotion was monitored using the same Ethovision software. The software defined grid lines that divided the entire field (100cm X 100cm) by a center/inner zone (30cm X 30cm) and a peripheral/outer zone being 20cm from each wall. An increased amount of time spent in the peripheral zone of the maze is related to greater anxiety (Prut and Belzung, 2003).

2.1.8. Rotarod Test

We used the Columbus ECONOMEX Rotarod (Columbus, Ohio) instrument. At 11 weeks of age, mice were placed on a rotating rod, which was set at a speed of 1 rotation per minute (RPM), with an acceleration of 1 rotation per minute every second (RPM/sec) for a maximum of 5 minutes. Each mouse underwent four trials with a one-minute rest period between each trial. The latency to fall on the first trial was used to assess motor coordination because the majority of mice remained on the bar for 5 minutes (maximum time) on the subsequent trials, resulting in a ceiling effect.

2.1.9. Forced Swim Test

A 4000ml glass beaker was filled with 3000ml of water at a temperature of $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$. At 11 weeks of age, each mouse was placed in the beaker and allowed to swim for 5 minutes.

Behaviour was recorded using the Panasonic WV-CP284 camera mounted on an Optex T165 tripod. The total time spent immobile was analyzed by two raters blind to experimental conditions with an inter-rater discrepancy within 10%. The duration of immobility on the Forced Swim Test (FST) is recognized as an indicator of behavioural despair (Porsolt et al., 1979).

2.1.10. Restraint Stress

Twenty-four hours after completing behaviour testing, the mice underwent the restraint stress test. Mice were placed into well-ventilated 50ml tubes and remained in the tubes for 30 minutes.

At the end of restraint, mice were returned to their home cage and left unhandled for another 30 minutes prior to euthanasia. Mice received an intraperitoneal injection of Euthanyl (prepared from Euthansol; Merck Animal Intervet Canada Corp; Kirkland, Quebec) 30 minutes post-restraint.

2.1.11. Tissue collection and immunohistochemistry (IHC)

Mice were perfused by cardiac puncture with 20ml of 0.9% saline followed by 20ml of 4% paraformaldehyde (PFA) to fix the tissue. Then, the PFA was replaced with 10ml of a 30% sucrose. Twenty-four hours later, the brains were transferred to conical tubes with 50ml of 30% sucrose solutions and stored at 4°C until sectioning. Brains were sliced using a Leica VT1200 S automated vibrating blade microtome at a thickness of 40 microns. Sections were stored in cryoprotectant at -20°C prior to immunostaining. Free-floating tissue sections were incubated for 30 minutes in a citric acid solution. Sections were rinsed with TBS and incubated for 30 minutes in a 0.1M Glycine antigen retrieval buffer. The sections were rinsed with TBS once again and incubated in a concentrated blocking solution containing TBS, 20% normal goat serum (NGS), 0.3% Triton-X, and 1% Hydrogen Peroxide for 30 minutes. Sections were then transferred to a solution containing a primary rabbit antibody specific to TLR4 (CAU27228 Biomatik; 1:400 in TBS, 2%NGS, 0.3% Triton-X) and incubated overnight at room temperature. The following day, sections were washed with TBS for 15 minutes to remove unbound antibodies. Tissue was incubated for 60 minutes at room temperature in a secondary antibody (Vector, BA-1000, Biotinylated Goat anti-rabbit IgG; PC38 Millipore 1:500) containing TBS, 2% NGS and 0.3% Triton-X. Tissue was then washed for 30 minutes with 0.2% Triton-X in TBS and incubated in the ABC detection system (Vector, PK-6100, ABC-Elite standard; Vector Laboratories, Burlingame, CA, USA; 1:100). After one hour, sections were washed for 30 minutes with TBS to

remove unbound antibodies and submerged in 3,3'-Diaminobenzidine (DAB, Vector, SK-4100; Vector Lab; 1:500) until color change. Finally, sections were rinsed for 15 minutes with TBS followed by distilled water. Tissue was stored at 4°C prior to mounting.

2.1.12. Image analysis and cell counting

The identification of the PVN was based on *The Mouse Brain Atlas in Stereotaxic Coordinates* (Franklin and Paxinos, 1997), plate 38 (Bregma -0.82mm). Brain sections were analyzed using a light microscope (Olympus BX51) that was interfaced with a scan camera (Jenoptik ProgRes MF; Jena, Germany). Pictures of the PVN were taken at 20X and were merged using 2010 Adobe Photoshop. The count was done on 5.1 Fiji Image J software created by the National Institute of Health. Manual cell counting was conducted using two-raters blind to experimental conditions and required a 20% agreement among final count.

2.2. Procedures

As of 5 weeks of age, mice (N = 160) were either exposed to milk control or to kefir. At 6 weeks of age, mice were treated with either saline or LPS. A subset of mice (n = 80) were euthanized 8 hours following treatment to examine peripheral cytokine concentrations and central cytokine mRNA expression (Fig. 1). The remaining mice (n = 80) were monitored for sickness behaviour over the next 48 hours. After recovery from sickness, mice continued consuming either milk control or kefir for one week (for a total two-week course of probiotic treatment). At 10 weeks of age, the mice began behavior testing with the EPM, OFT, Rotorod, and FST (Fig. 1). Each behavior test was followed by a 48-hour rest period. Forty-eight hours after the final behaviour test, mice were exposed to a 30-minute restraint stress test and were euthanized 30 minutes following the termination of restraint.

2.3 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (version 22). A three-way mixed analyses of variance (ANOVA) was used to examine the effect of sex (male or female), LPS treatment (saline or LPS) and probiotic treatment (milk control or kefir) on sickness response (as published in Cai et al., 2016; Girard-Joyal & Ismail, 2017; Kolmogorova et al., 2019) and relative abundance of bacterial phyla over time. Three-way between-subject ANOVAs were used to examine treatment effects of sex, LPS treatment and probiotic treatment on peripheral cytokine concentration, central cytokine mRNA expression, duration of immobility in the FST, time spent in the inner zone of the OFT, time spent in the open arms and distance travelled in the EPM, latency to fall on the rotorod test, and TLR4 expression in the PVN of the hypothalamus following restraint stress. Statistically significant effects were followed by pairwise comparisons using the Bonferroni correction, when appropriate. A Pearson bivariate correlation was used to assess the predictive relationship between TLR4 expression and time spent in the open arms on the EPM. For all tests, the criterion for statistical significance was set to $p < 0.05$.

3. Results

3.1 Pubertal probiotic treatment reduced sickness behaviour in males and females in a time-specific manner and reduced LPS-induced decreases in body weight in females.

To test if probiotics would decrease sickness outcomes caused by an immune challenge, we began exposing mice to a probiotic culture or control for one week prior to LPS treatment and measured sickness behaviour and body weight for up to 48 hours post-injection. Three-way repeated measures ANOVA revealed main effects of sex ($F_{(1,108)} = 62.005, p < .001, \eta^2_p = 0.365$) and LPS treatment ($F_{(1,108)} = 1823.776, p < .001, \eta^2_p = 0.944$) on sickness behaviour. There was also a significant sex x LPS treatment interaction ($F_{(1, 108)} = 40.139, p < .001, 271, \eta^2_p = 0.271$), and time X probiotic treatment X LPS treatment ($F_{(2, 108)} = 3.378, p = .038, 271, \eta^2_p = 0.059$).

Pairwise comparisons showed that LPS-treated male mice exposed to kefir ($M = .313$, $SE = .115$) displayed less sickness behaviour than the milk control condition ($M = .700$, $SE = .103$) at 48 hours following LPS exposure (Mean difference (MD) = 0.387, standard error (SE) = 0.154, $p < .001$) (Fig. 2a).

Female LPS-treated mice receiving milk control ($M = 2.85$, $SE = .176$) displayed significantly more sickness behavior than those receiving kefir ($M = 1.625$, $SE = .161$) at 12 hours following exposure to immune challenge ($p < .001$) (Fig. 2b). Mice treated with saline did not vary significantly in sickness behavior across probiotic conditions. There were also main effects of probiotic treatment ($F_{(2,55)} = 11.535$, $p < 0.001$) and LPS treatment ($F_{(1,55)} = 25.286$, $p < 0.001$, $\eta^2_p = 0.315$), and a significant probiotic treatment $\times$ LPS treatment interaction ($F_{(2, 55)} = 3.265$, $p = 0.046$, $\eta^2_p = 0.106$) on percent body weight change 48 hours after LPS injection. Pairwise comparisons revealed that, in mice not consuming probiotic, LPS-induced significant reductions in body weight in both male ($M = -6.545$, $SE = 1.503$) and female ($M = -9.996$, $SE = 1.779$) mice compared to saline-injected controls ($M = -1.232$, $SE = 1.624$; $M = -4.295$, $SE = 1.624$, respectively) (MD = 5.313, $SE = 2.515$, $p < 0.05$; MD = 5.70, $SE = 2.408$, $p < 0.05$, respectively). Probiotic treatment blocked LPS-induced weight loss in males, as no significant difference was observed between LPS-treated male mice and saline controls at 48 hours post-injection. LPS-treated female mice that received kefir probiotic treatment ($M = -778$, $SE = 1.779$) lost significantly less weight as compared to the milk control group ($M = -9.996$, $SE = 1.779$) (MD = 9.217, $SE = 2.515$, $p < 0.05$) (Fig. 3).

A three-way ANOVA revealed main effects of sex ($F_{(1, 71)} = 28.647$, $p < 0.001$, $\eta^2_p = 0.287$) and probiotic treatment ($F_{(1, 71)} = 5.404$, $p < 0.023$, $\eta^2_p = 0.071$) on percent body weight change across two weeks of probiotic treatment. Pairwise comparisons revealed that, regardless

of probiotic treatment, LPS-treated males gained more weight ($M = 21.691$, $SE = 2.545$; $M = 25.949$, $SE = 2.276$, respectively) than LPS-treated females ($M = 10.335$, $SE = 2.078$; $M = 13.634$, $SE = 2.276$, respectively) ($p = 0.001$; $p < 0.001$, respectively). Saline-treated males ($M = 17.667$, $SE = 2.276$) gained more weight than saline-treated females ($M = 10.880$, $SE = 2.078$) in the kefir condition ($p = 0.044$). A main effect of sex ($F_{(1, 71)} = 71.394$, $p < 0.001$, $\eta^2_p = 0.437$) on body weight change from start of probiotic treatment to adulthood was observed. Males gained more weight than females across all treatment conditions ($p < 0.02$). No other treatment differences in body weight were observed.

3.2 Probiotic treatment prevented LPS-induced increases in both pro- and anti-inflammatory peripheral cytokines, in a sex-specific manner.

We further investigated if probiotic treatment would reduce the peripheral inflammatory response to LPS. Thus, we next examined pro- and anti-inflammatory peripheral blood serum cytokine concentrations in responses to LPS treatment at 8 hours post-infection. Three-way ANOVA revealed main effects of sex on interleukin-6 (IL-6) ($F_{(1,62)} = 12.86$, $p < 0.001$, $\eta^2_p = 0.172$) and interleukin-10 (IL-10) ($F_{(1,58)} = 5.43$, $p = 0.023$, $\eta^2_p = 0.086$). A main effect of LPS treatment was found on IL-6 ($F_{(1,62)} = 202.59$, $p < 0.001$, $\eta^2_p = 0.766$), IL-10 ($F_{(1,58)} = 131.78$, $p < 0.001$, $\eta^2_p = 0.694$), interleukin-12 (IL-12) ($F_{(1,60)} = 28.54$, $p < 0.001$, $\eta^2_p = 0.322$), tumour necrosis factor α (TNF α) ($F_{(1,59)} = 50.97$, $p < 0.001$, $\eta^2_p = 0.464$) and interferon- γ (IFN γ) ($F_{(1,61)} = 84.31$, $p < 0.001$, $\eta^2_p = 0.580$). A significant sex X LPS treatment interaction was found for IL-6 ($F_{(1,62)} = 12.68$, $p < 0.001$, $\eta^2_p = 0.170$) and IL-10 ($F_{(1,58)} = 5.12$, $p < 0.027$, $\eta^2_p = 0.081$). A significant sex X probiotic treatment ($F_{(1,60)} = 5.12$, $p = 0.027$, $\eta^2_p = 0.079$) and a sex X probiotic treatment X LPS treatment interactions ($F_{(1,60)} = 5.04$, $p = 0.028$, $\eta^2_p = 0.078$) was found for interleukin-12 (IL-12).

All LPS-treated male and female mice showed significantly higher concentrations of IL-6, IL-10, IL-12, TNF α and IFN γ compared to saline-treated counterparts ($p < 0.05$) (Table 2). Pairwise comparisons revealed that LPS-treated males showed greater concentration of IL-6 compared to females in both the kefir and milk control conditions (MD = 2762.71, SE = 969.33, $p = 0.006$ and MD = 4531.39, SE = 1111.89, $p < 0.001$, respectively) (Fig. 4a). LPS-treated males showed higher concentrations of IL-10 (MD = 229.06, SE = 74.39, $p = 0.003$) (Fig. 4b) and IL-12 (MD = 97.60, SE = 27.28, $p = 0.001$) (Fig. 4c) compared to females, but only in the milk control group. These sex differences were eliminated with kefir treatment. LPS-treated males exposed to kefir showed a lower concentration of IL-10 compared to LPS-treated males who received milk control treatment (MD = 177.222, SE = 68.584, $p = 0.012$) (Fig. 4b). Moreover, LPS-treated males in the milk control condition showed higher concentration of IL-12 compared to saline controls (MD = 137.422, SE = 25.148, $p < 0.001$), but this effect was eliminated with kefir treatment (Fig. 4c). LPS-treated males exposed to kefir treatment displayed lower concentrations of IL-12 (MD = 99.58, SE = 25.148, $p < 0.001$) (Fig. 4c) and TNF α (MD = 59.95, SE = 21.30, $p < 0.007$) (Fig. 4d) compared to LPS-treated males who received milk control treatment. In females, concentrations of IL-6, IL-10, IL-12, TNF α and IFN γ did not differ between probiotic and control treatment (Table 2).

3.3 Probiotic treatment reduced LPS-induced increases in central cytokine mRNA expression in a sex-specific manner.

We next assessed brain cytokine mRNA expression in three regions (hippocampus, hypothalamus and prefrontal cortex) to further investigate the effect of probiotics on LPS-induced central inflammatory response. Three-way ANOVA revealed main effects of sex ($F_{(1,34)}=5.281$, $p = 0.028$, $\eta_p^2 = 0.134$) and LPS treatment ($F_{(1,34)}=13.62$, $p = 0.001$, $\eta_p^2 =$

0.286), and a significant sex x LPS treatment interaction ($F_{(1,34)} = 5.35, p = 0.027, \eta^2_p = 0.136$) on IL-1 β mRNA expression in the hypothalamus. A main effect of LPS treatment ($F_{(1,35)} = 10.17, p = 0.003, \eta^2_p = 0.225$) on IL-1 β mRNA expression was found in the hippocampus. A main effect of LPS treatment ($F_{(1,35)} = 17.54, p < 0.01, \eta^2_p = 0.334$) and a significant sex x LPS treatment interaction ($F_{(1,35)} = 9.25, p = 0.004, \eta^2_p = 0.209$) was found on IL-1 β mRNA expression in the prefrontal cortex (PFC). Three-way ANOVA revealed main effects of sex ($F_{(1,38)} = 8.339, p = 0.006, \eta^2_p = 0.180$) and LPS treatment ($F_{(1,38)} = 25.74, p < 0.01, \eta^2_p = 0.404$), and significant sex x LPS treatment interaction ($F_{(1,38)} = 6.218, p = 0.017, \eta^2_p = 0.141$) on TNF α mRNA expression in the hypothalamus. A main effect of LPS treatment ($F_{(1,36)} = 12.37, p = 0.01, \eta^2_p = 0.256$) and a significant probiotic treatment x LPS treatment interaction ($F_{(1,34)} = 6.467, p = 0.015, \eta^2_p = 0.152$) were found for TNF α mRNA expression in the hippocampus. Main effects of sex ($F_{(1,34)} = 5.913, p = 0.020, \eta^2_p = 0.148$) and LPS treatment ($F_{(1,34)} = 11.639, p = 0.002, \eta^2_p = 0.255$), and significant sex x LPS treatment interaction ($F_{(1,34)} = 7.028, p = 0.012, \eta^2_p = 0.171$) were found for TNF α mRNA expression in the PFC. Three-way ANOVA revealed a main effect of LPS treatment on IL-6 mRNA expression in the hypothalamus ($F_{(1,38)} = 16.92, p < 0.01, \eta^2_p = 0.308$). A main effect of LPS treatment on IL-6 mRNA expression in the hippocampus ($F_{(1,34)} = 15.49, p < 0.01, \eta^2_p = 0.313$). A main effect of LPS treatment on IL-6 mRNA expression in the prefrontal cortex ($F_{(1,31)} = 16.41, p < 0.01, \eta^2_p = 0.346$).

Regardless of probiotic treatment, LPS-treated females showed more IL-1 β expression in the hypothalamus ($M = 23.117, SE = 4.430; M = 17.444, SE = 4.853$, respectively) ($p < 0.05$) (Fig. 5a) and the hippocampus ($M = 36.288, SE = 8.514; M = 27.665, SE = 8.514$, respectively) ($p = 0.037$) (Fig. 5b), and TNF α mRNA expression in the hypothalamus ($M = 5.666, SE = 1.065; M = 6.428, SE = 1.065$, respectively) ($p < 0.01$) (Fig. 5d) compared to saline-injected controls.

LPS-injected females also showed more IL-1 β in the PFC ($M = 38.096$, $SE = 5.312$) compared to saline injected controls ($M = .224$, $SE = 5.312$) ($p < 0.01$) (Fig. 5c) in the non-probiotic condition only. Probiotic consumption significantly reduced LPS-treated females IL-1 β mRNA expression in the PFC ($M = 14.876$, $SE = 5.312$) ($p = 0.004$) (Fig. 5c), and TNF α mRNA expression in the hippocampus ($M = 1.318$, $SE = .666$) ($p = 0.015$) (Fig. 5e) and PFC ($M = 7.406$, $SE = 2.836$) ($p = 0.023$) (Fig. 5f) compared to their non-probiotic counterparts. Regardless of probiotic treatment, LPS-treated females showed more IL-6 expressions in the hypothalamus ($M = 6.644$, $SE = 2.018$; $M = 9.068$, $SE = 1.842$, respectively) ($p < 0.05$) (Fig. 5g), the hippocampus ($M = 8.056$, $SE = 2.369$; $M = 9.303$, $SE = 2.163$, respectively) ($p = 0.009$) (Fig. 5h) compared to saline-injected controls. LPS-treated males in the milk control condition showed more IL-6 mRNA expression in the hippocampus ($M = 7.792$, $SE = 2.369$) ($p = 0.021$) and hypothalamus ($M = 5.607$, $SE = 1.842$) ($p = 0.05$) (Fig. 5h), compared to saline-injected controls ($M = .070$, $SE = 2.369$; $M = .347$, $SE = 1.842$, respectively). These differences in the males were eliminated in probiotic-treated groups. LPS-treated females that were not given probiotic treatment showed more TNF α mRNA expression in the hippocampus ($M = 3.714$, $SE = .666$) ($p < 0.02$) (Fig. 5e) and in the hypothalamus ($M = 5.66$, $SE = 1.065$) ($p < 0.05$) (Fig. 5d) compared to saline-injected controls ($M = .397$, $SE = .608$; $M = .960$, $SE = .972$, respectively). Likewise, LPS-treated males that were not given probiotic treatment showed greater TNF α mRNA expression in the hippocampus ($M = 2.668$, $SE = .608$) ($p = 0.001$) (Fig. 5e) and in the hypothalamus ($M = 3.275$, $SE = .972$) ($p = 0.04$) (Fig. 5d) compared to saline-injected controls ($M = .525$, $SE = .608$; $M = .345$, $SE = .972$, respectively). LPS-treated females that were not given probiotics also showed more TNF α mRNA expression in the PFC ($M = 16.952$, $SE = 2.836$) compared to saline controls ($M = .268$, $SE = 2.836$) ($p = 0.001$) and their LPS-treated male counterparts ($M = 7.406$, $SE = 2.836$) ($p =$

0.002) (Fig 5f). These sex and LPS-induced differences were eliminated with probiotic treatment.

3.4 Probiotic consumption prevented LPS-induced changes to the gut microbiome, but in a sex-specific manner.

To elucidate changes in gut microbiota as a result of LPS treatment, we analyzed the relative abundance of each bacterial phylum as a percentage to the total phylum identification based upon 16S rRNA sequencing. Three-way ANOVA revealed a significant main effect of time on the relative abundance (%) of Firmicutes ($F_{(5,75)} = 7.624, p < .001, \eta^2_p = 0.337$), Bacteroidetes ($F_{(5,75)} = 7.624, p < .001, \eta^2_p = 0.348$) and Proteobacteria ($F_{(5,70)} = 5.842, p = .017, \eta^2_p = 0.294$) phyla. A significant main effect of LPS treatment was found on relative abundance of Firmicutes ($F_{(1,15)} = 10.725, p = .005, \eta^2_p = 0.417$) and Proteobacteria ($F_{(1,14)} = 8.885, p = .01, \eta^2_p = 0.388$) phyla. A significant time x sex interaction was found on relative abundance of Firmicutes ($F_{(5,73)} = 5.754, p = .001, \eta^2_p = 0.227$), Bacteroidetes ($F_{(5,75)} = 12.332, p = .001, \eta^2_p = 0.468$) and Proteobacteria ($F_{(5,75)} = 4.81, p = .029, \eta^2_p = 0.256$) phyla. A significant time x LPS treatment interaction was observed on the relative abundance of Firmicutes ($F_{(5,75)} = 4.227, p = .002, \eta^2_p = 0.220$), Bacteroidetes ($F_{(5,75)} = 2.790, p < .001, \eta^2_p = 0.116$) and Proteobacteria ($F_{(5,75)} = 5.137, p = .024, \eta^2_p = 0.268$) phyla. A significant probiotic treatment x LPS treatment interaction was observed on the relative abundance of Verrucomicrobia ($F_{(1,14)} = 4.534, p = .051, \eta^2_p = 0.245$). A significant time x sex x LPS treatment interaction was observed on the relative abundance of Proteobacteria phylum ($F_{(5,75)} = 5.146, p = .024, \eta^2_p = 0.269$) and a significant time x sex x probiotic treatment x LPS treatment interaction was found on relative abundance of Bacteroidetes phylum ($F_{(5,75)} = 3.433, p = .008, \eta^2_p = 0.197$).

No significant difference in bacterial phylum was observed between the groups at 5 weeks old prior to probiotic treatment. After one week of kefir probiotic treatment, at 6 weeks old and prior to LPS treatment, males showed a higher percentage of Bacteroidetes phylum ($M = 79.827$, $SE = 9.512$) compared to females ($M = 47.733$, $SE = 7.766$) ($p = .02$) (Supplementary Fig. 1). At 24 hours post-LPS treatment, LPS-treated males exposed to the milk control ($M = 39.4851$, $SE = 8.055$) show lower percentage of Bacteroidetes phylum compared to their saline counterparts ($M = 67.258$, $SE = 5.696$) ($p = .014$). LPS-treated males showed higher percentage of Proteobacteria phylum ($M = 18.33$, $SE = 2.187$) compared to LPS-treated females ($M = 2.387$, $SE = 1.263$) ($p = .001$) and their saline-treated male counterparts ($M = .359$, $SE = 1.546$) ($p = .001$) in the milk control condition. Similarly, LPS-treated females in the milk control condition show lower percentage of Firmicutes phylum ($M = 7.205$, $SE = 9.147$) ($p = .026$) but higher percentage of Bacteroidetes phylum ($M = 88.913$, $SE = 6.482$) ($p = .012$) compared to saline-treated counterparts ($M = 36.322$, $SE = 7.469$; $M = 64.416$, $SE = 5.567$, respectively). Treatment differences were eliminated in the mice that consumed the probiotic. Finally, LPS-treated males exposed to kefir showed a significantly lower percentage of Proteobacteria phylum ($M = 3.797$, $SE = 1.093$) compared to their milk-treated counterparts ($M = 18.333$, $SE = 2.187$) ($p = .001$) (Fig. 6).

At the end of the two-week probiotic treatment, pairwise comparisons revealed that both LPS- and saline-treated males display lower levels of Firmicutes phylum ($M = 20.712$, $SE = 6.809$; $M = 18.034$, $SE = 9.629$, respectively) compared to female counterparts ($M = 54.133$, $SE = 9.629$; $M = 48.331$, $SE = 7.862$, respectively) ($p = .025$; $p = .028$, respectively), when treated with milk control. LPS-treated males that were exposed to the milk control also showed lower percentage of Verrucomicrobia phylum ($M = .004$, $SE = 1.418$) compared to their saline

counterparts ($M = 6.600$, $SE = 1.418$) ($p = .005$) and kefir-treated male counterparts ($M = 3.174$, $SE = 1.158$) ($p = .04$). At 10 weeks of age (in adulthood), both LPS- and saline-treated males show lower levels of Firmicutes ($M = 29.143$, $SE = 6.369$; $M = 12.405$, $SE = 6.369$, respectively) compared to their female counterparts ($M = 57.085$, $SE = 6.369$; $M = 50.565$, $SE = 5.200$, respectively) ($p = .007$; $p = .001$, respectively) in the milk condition. Moreover, in the milk condition, saline-treated males showed higher levels of Verrucomicrobia ($M = 16.461$, $SE = 1.518$) compared to saline-treated females ($M = .005$, $SE = 1.073$) ($p = .001$). No sex difference was observed at this time point when mice were treated with kefir (Supplementary Fig. 1).

3.5 Pubertal probiotic treatment prevented LPS-induced depression-like behaviour in female mice.

To assess the enduring effects of LPS and probiotic treatment, we next examined depression-like behaviour using the forced swim test (FST). Duration of immobility was assessed in male and female mice on the FST at 10 weeks of age. Three-way ANOVA revealed significant sex $\times$ probiotic treatment interaction ($F_{(1, 67)} = 9.843$; $p < 0.005$, $\eta^2_p = .128$) on time immobile. Pairwise comparisons revealed that LPS-treated females ($M = 82.950$, $SE = 9.767$) spent more time immobile compared to saline-injected controls ($M = 53.600$, $SE = 9.767$) when treated with milk control (MD = 29.35, $SE = 13.812$, $p < 0.05$) (Fig. 7b). Saline-treated females that received kefir probiotic treatment ($M = 91.200$, $SE = 9.767$) also spent more time immobile compared to milk controls (MD = 37.60, $SE = 13.812$, $p < 0.005$). LPS-treated males that received kefir probiotic treatment ($M = 58.187$, $SE = 2.078$) spent less time immobile compared to milk controls ($M = 87.45$, $SE = 9.767$) (MD = -29.262, $SE = 14.65$, $p = 0.05$) (Fig. 7a).

3.6 Pubertal probiotic treatment prevented LPS induced anxiety-like behaviour and increased stress reactivity in male mice.

The next objective was to examine enduring effects of LPS and probiotic treatments on enduring anxiety-like behaviour and stress reactivity. Time spent in the inner zone of the OFT was analyzed in adult male and female mice. Statistical analyses revealed significant main effects of sex ($F_{(1, 97)} = 4.742, p < .05$) and LPS treatment ($F_{(1, 97)} = 5.504, p < .05$). Pairwise comparisons revealed that LPS-injected male mice in the milk control ($M = 19.019, SE = 2.513$) condition spent less time in the inner zone compared to saline-injected counterparts ($M = 27.719, SE = 2.810$) ($MD = -8.700, SE = 3.770, p = .023$) (Fig. 8a). Time spent in the open arms of the elevated plus maze (EPM) in adult male and female mice was also analyzed and statistical analyses revealed main effects of sex ($F_{(1, 102)} = 11.040, p < .005$) and LPS treatment ($F_{(1, 102)} = 5.535, p < .05$) on the time spent in the open arms. Pairwise comparison revealed that, in the milk control condition, LPS-treated males ($M = 39.476, SE = 7.965$) spent less time in the open arms compared to saline-injected controls ($M = 69.974, SE = 9.339$) ($MD = -30.498, SE = 12.274, p < 0.05$) (Fig. 8c). In the kefir probiotic condition, saline-treated males ($M = 60.984, SE = 8.353$) spent more time in the open arm compared to saline-treated females ($M = 27.421, SE = 8.353$) ($MD = 33.649, SE = 12.137, p < 0.05$) (Fig. 8c).

Motor performance was also assessed. While sex affected motor performance, no other treatment differences were observed. There was a sex x probiotic x treatment interaction ($F_{(2, 105)} = 3.102, p < .05$) on distance travelled in the EPM. Pairwise comparisons revealed that saline-injected males ($M = 1963.137, SE = 74.287$) travelled a greater distance compared to saline-injected females ($M = 1645.006, SE = 74.287$), in the kefir probiotic condition ($MD = 318.131, SE = 105.057, p < 0.005$). The rotorod test assessed the latency to fall in males and females. Statistical analyses revealed no significant main effects or interactions. Other locomotion measures included zone transitions and travel velocity in the OFT. A main effect of sex ($F_{(1, 68)} =$

16.374, $p = .001$) was observed in zone transitions. Pairwise comparisons revealed that in the milk control condition, saline-treated males ($M = 22.250$, $SE = 2.227$) had greater zone transitions compared to saline-treated females ($M = 11.700$, $SE = 1.992$) ($MD = 10.55$, $SE = 2.99$, $p = .001$). No other main effects of interactions were observed (Supplementary Fig. 2).

3.7 Pubertal probiotic treatment blocks LPS-induced programming of TLR4 expression in the PVN following exposure to a novel stressor in adulthood.

TLR4 plays a central role in the innate immune response (Akira, Takeda, Kaisho, 2001; Takeda, 2004) and research suggests that TLR4 directly contributes to the development of neuroinflammatory diseases (Walter et al., 2007; Buchanan et al., 2010). We examined TLR4 expression in the PVN of the hypothalamus because this region is directly involved in the stress and immune responses (Dange et al., 2015; Tang et al., 2017). Statistical analyses revealed significant main effects of sex ($F_{(1,34)} = 4.955$, $p = .033$, $\eta^2_p = 0.127$) and LPS treatment ($F_{(1,34)} = 9.361$, $p = .004$, $\eta^2_p = 0.216$) on TLR4 expression. Pairwise comparisons revealed that LPS-treated male mice ($M = 52.500$, $SE = 7.576$) showed significantly greater TLR4 expression compared to saline-treated males ($M = 29.800$, $SE = 7.576$), in the milk control condition ($MD = 22.70$, $SE = 10.714$, $p = .042$) (Fig. 9c). Treatment differences were absent in male mice that consumed probiotic. No treatment difference was observed in females. Statistical analyses revealed a negative correlation between TLR4 cell expression and time spent in the open arms of the EPM ($r = -.649$, $p = 0.043$), but only in males (Fig. 9e). No significant correlation was found between TLR4 cell expression and time spent in the open arms of the EPM in males treated with kefir.

4. Discussion

Several studies to date have reported enduring detrimental effects of an immune challenge during puberty (Olesen et al., 2011; Ismail & Blaustein, 2013; Ismail, Kumlin & Blaustein, 2013; Girard-Joyal et al., 2017; Kolmogorova et al., 2019). Moreover, a growing body of research suggests the gut microbiome plays an important role during prenatal periods in development on enduring brain functioning (Diaz Heijtz et al., 2011; Borre et al., 2014; de Theije et al., 2014; Jašarević et al., 2018). However, the influence of gut microbiota during puberty, a critical period in development, against stress-induced outcomes on brain functioning and emotional behaviour in adulthood was unknown. The aim of the present study was to examine sex-specific effects of gut manipulation with probiotics during puberty against LPS-induced inflammatory and microbial outcomes and enduring effects on anxiety- and depression-like behaviours, stress reactivity and on associated neurochemical changes.

LPS is a well-recognized bacterial endotoxin, which activates a robust immune response (Beaty et al., 1994; de Bont et al., 1998). As expected, all mice treated with LPS displayed more sickness behaviour than saline controls. Male mice displayed greater and more prolonged sickness behavior compared to female mice, which replicates previous findings from our laboratory (Cai et al., 2016). We hypothesized that probiotics would reduce the inflammation and sickness behavior caused by the LPS treatment. Our findings support our hypothesis and establish that probiotics accelerate recovery from sickness in both males and females in a time-specific manner. Specifically, probiotic treatment decreased LPS-induced sickness behaviour in males at 48 hours and in females at 12 hours following exposure to immune challenge. This is in line with previous work finding positive effects of probiotics on immune response and resulting in reduced sickness behaviours in mice following liver inflammation (D'Mello et al., 2015).

There were no significant changes in body weight for male mice. Therefore, probiotics are more effective against LPS-induced weight loss and sickness in females.

Exposure to LPS causes an increase in serum cytokine production, including IL-6, TNF α , IL-1 β , IFN γ , IL-10, and IL-12 (Correa et al., 2007; Kim et al., 2007; Cai et al., 2016) and probiotics are associated with decreased production of pro-inflammatory cytokines (O'Mahony et al., 2005; Hemarajata & Versalovic 2013; D'Mello et al., 2015; Schachter et al., 2018). Overall, at 8 hours post-LPS exposure, males showed greater peripheral cytokine concentration compared to female mice. Interestingly, at this same time point, females displayed much greater central IL-1 β and TNF α mRNA expression in the hypothalamus and PFC compared to male counterparts. These results are consistent with other findings which also show sex differences in LPS-induced cytokines between the periphery and the CNS (Sharma et al., 2018), indicating that peripheral inflammatory responses are not always consistent with those seen centrally (Mouihate et al., 2010). Probiotic treatment reduced peripheral cytokine concentrations of IL-10, IL-12 and TNF α in males at 8 hours following LPS exposure. Centrally, LPS increased IL-6 and TNF α mRNA expression in the hypothalamus and hippocampus in these males. These treatment differences were eliminated in males that consumed probiotics. This suggests that probiotic exposure reduces central and peripheral LPS-induced inflammation in males. Females had a much stronger central inflammatory response compared to males. Yet, these sex differences were eliminated with probiotic treatment. Specifically, females showed a reduced inflammatory response in the PFC and hippocampus with probiotic treatment. Reduced inflammatory response from probiotics may be tied to increased production of a metabolite of dietary tryptophan, which can modulate and reduce the inflammatory status of astrocytes and exert downstream effects on neuroinflammation (Rothhammer et al., 2016; Zelante et al., 2013). Moreover, probiotics such as

bifidobacterium and *lactobacillus* are shown to exert antibacterial effects on pathogenic bacteria (Collado et al., 2007; Servin, 2004) and *lactobacillus* can enhance the intestinal barrier (Mack et al., 2003), which could reduce pathogen-induced inflammation. Overall, our findings establish that probiotics accelerate recovery from sickness and reduce the intensity of the inflammatory response following an immune challenge during puberty. These sex-specific responses may help explain the mechanisms influencing enduring behavioural and neurochemical changes.

While there is great diversity in the function of each taxonomic phyla (Jandhyala, 2015), we were interested in overall changes to gut microbiota profiles, as measured by the relative abundance and diversity in the composition of bacterial phyla. In the current study, we hypothesized that LPS treatment, and the resulting inflammation, would induce significant changes in the gut microbiome and exposure to probiotics would reduce these LPS-induced changes in bacterial phyla. As expected, LPS treatment induced changes in the gut microbial profiles. However, kefir treatment eliminated sex differences and prevented LPS-induced changes in the relative abundance of gut microbial phyla. We suggest that kefir is preventing stress-induced microbial fluctuations and protecting the structural integrity of the gut mucosal barrier (Demaude, 2006; Söderholm & Perdue, 2001), resulting in a reduced inflammatory response during puberty.

We also hypothesized that pubertal LPS exposure would induce enduring effects on anxiety- and depression-like behaviours and increase stress reactivity in all mice. Indeed, probiotic consumption during this critical stage in development had important long-term modulating effects on behaviour into adulthood. LPS-induced depression-like behaviour in females while LPS-treated males showed more anxiety-like behaviour and stress reactivity. These results suggest that pubertal exposure to an immune challenge has detrimental effects on

both sexes, but likely affect different pathways. Pubertal probiotic treatment prevented stress-induced depression-like behaviour in females by eliminating treatment differences between immune- and non-immune challenged female mice. These findings are consistent with previous literature showing that probiotic consumption has beneficial effects on emotional behaviors such as anxiety- and depression-like behaviours and stress-induced CORT responses. Specifically, male mice fed *Lactobacillus rhamnosus* for 28 days had shown reduced stress-induced corticosterone and anxiety- and depression-like behaviors (Bravo et al., 2011). Our findings strengthen these observations and highlight the enduring effects of probiotics during the pubertal period. Saline control mice treated with probiotics displayed subtle non-significant changes in anxiety- and depression-like behaviors in both sexes. We have noted this effect in other unpublished findings from our laboratory and believe that these effects are due to the impact of probiotics on other aspects of behaviour, such as subtle effects on gross motor performance. These observations confirm that probiotics do not cause any detrimental effects on behaviour. Thus, our saline group exposed to kefir is a more representative control comparison to our LPS-treated kefir group on anxiety- and depression-like behaviours.

Exposure to an immune challenge during puberty increased the HPA axis responsiveness to a novel heterotypic stressor (restraint) in adulthood in males. We examined TLR4 expression in the PVN following stress exposure because this signaling pathway is directly involved in stress-induced visceral hypersensitivity (Chen et al., 2015) and the innate immune response (Akira, Takeda, Kaisho, 2001; Takeda, 2004). We hypothesized that exposure to pubertal immune challenge would result in significant upregulation of TLR4 receptor expression. Manipulation of gut microbiota through probiotics during puberty was expected to mitigate this effect. Our findings point to an important mechanistic influence of TLR4; probiotics prevented

LPS-induced anxiety-like behavior by blocking the increase in TLR4 expression in the PVN. As this effect was only seen in males, our data highlight an enduring sex difference in TLR4 expression following pubertal LPS exposure. Males naturally have greater expression of TLR4 receptors on peritoneal macrophages compared to females (Marriott et al., 2006). This sex difference is likely due to estradiol suppressing immune response (Razmara et al., 2007), modulating microglial inflammatory responses (Baker et al., 2004) and causing TLR4 downregulation (Moeinpour et al., 2007, Hsieh et al., 2007). This sex difference could also be due to varying concentrations of estradiol depending on the stage of estrus cycle in females. Nonetheless, these sexual dimorphisms likely prevented LPS-induced increase in TLR4 expression in females. Our work shows that pubertal immune challenge affects different neural circuits in males and females. These long-lasting effects on TLR4 expression also suggest important programming effects of probiotics, which could involve a variety of mechanisms. Taken together, we speculate that probiotics exerted beneficial effects on the microbial environment which modulated LPS-induced TLR4 programming to novel stressors in adulthood.

The kefir preparation required growth in skim milk. Given that milk provides additional nutrients through lipids, lactose, vitamins, and protein (Jost, 2007), we chose to expose our control mice to the same skim milk to minimize the confounding effects of these nutritional benefits. In previous publications from our laboratories, we tested the enduring effects of LPS in CD1 mice exposed to a normal water diet (Girard-Joyal et al., 2015; Girard-Joyal & Ismail, 2017; Ismail, Kumlin & Blaustein, 2013; Laroche et al., 2009; Olesen et al., 2011). We observed similar effects of LPS in our milk controls compared to these studies. However, we acknowledge that minor differences between a milk and water control are possible. Second, the aim of the current study was to elucidate mitigating effects of probiotics during the pubertal stress-sensitive

period. Beneficial effects of probiotics on behaviour have been suggested in a number of studies (Baharav et al., 2004; Bercik et al., 2010; Bravo et al., 2011; Chung et al., 2014; Park et al., 2018; Messaoudi et al., 2011; Rao et al., 2009). Therefore, it is likely that probiotics would exert beneficial effects at other ages. Nonetheless, the current work highlights the importance of the gut microbiome during this critical period of development against LPS-induced immediate immune response and long-term brain activation and behavioural outcomes in adulthood.

5. Conclusions

This study examined sex-specific outcomes in the combined effects of pubertal immune challenge and probiotic treatment on acute immune response and inflammation, and enduring effects on depression- and anxiety-like behaviors and stress-reactivity in adulthood. The results show that pubertal probiotic exposure modulates LPS-induced depression-like behavior in female mice and anxiety-like behaviour and stress-reactivity in male mice. However, pubertal probiotic treatment prevented these LPS-induced impairments later in life by altering the gut microbial composition, reducing acute inflammatory responses, and blocking stress-induced increases in TLR4 expression in the PVN. This work highlights that the gut microbiome plays a central role in modulating lasting responses to pubertal immune challenge, in a sex-specific manner. Research is just beginning to elucidate the effects of the gut microbiome on behaviour and neurophysiology, and even less is known about its role in neurodevelopment during puberty, a critical period with a high emergence of psychiatric disorders. The findings of the current study add to our understanding of the vulnerabilities during pubertal development and the microbial impact on neurobehavioral outcomes later in life.

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AUTHOR CONTRIBUTIONS

N. Ismail and E. Murray designed the experiment and co-wrote the manuscript. R. Sharma, K. Mar, M. Lukasik, R. Barve, A. Pirwani, E. Malette-Guyon, S. Lamba, B. Thomas, H. Sadeghi-Emamchaie, contributed in data collection. Jacky Liang for training and technical support. E. Murray analyzed the data and created figures. K. Smith created figure 6 and performed biostatistics on the gut microbial content. J. Mallet and C. Matar provided the equipment and support of the multiplex Luminex immunoassay.

COMPETING FINANCIAL INTEREST

The authors declare no competing financial interests or potential conflicts of interest.

Target Gene	Forward	Reverse
β -actin	GAACCCTAAGGCCAACCGTG	GGTACGACCAGAGGCATACAGG
IL-1 β	TCTTGGGACTGATGCTGGTG	CAGAATTGCCATTGCACA ACTC
TNF α	GCCTATGTCTCAGCCTCTTCTC	GCCATTGCGGA ACTTCTCATCC
IL-6	GCCTTCTTGGGACTGATGCT	GCCATTGCACA ACTCTTTTCTC

Table 1. Summary of Primer Sequence for real-time qPCR reaction.

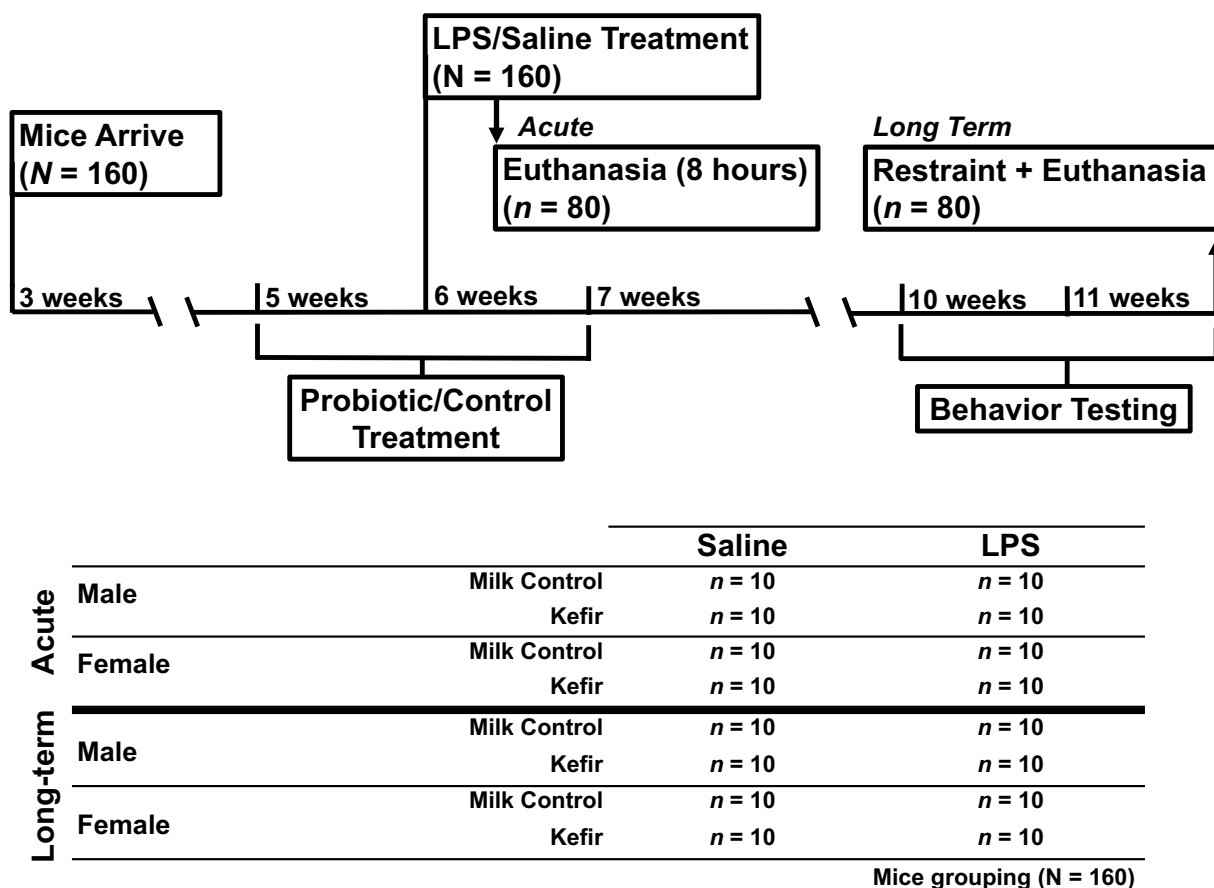


Figure 1. Experimental timeline (A) and groups and sample size (B) of mice examined for LPS-induced immune response 8 hours post-injection (acute effects) and the enduring effects of pubertal LPS exposure and probiotic treatment (long term effects).

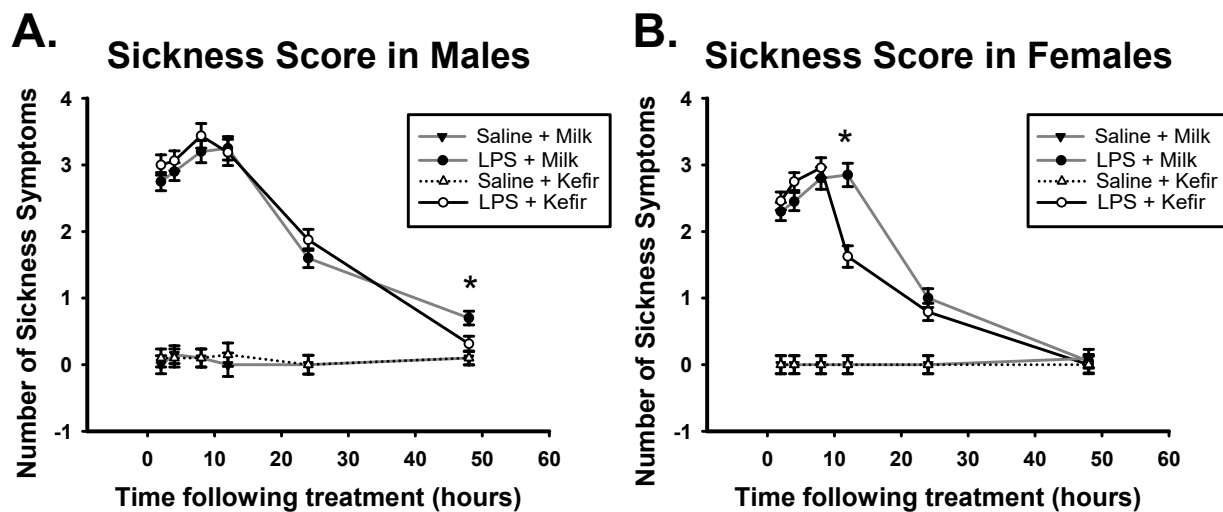


Figure 2. Sickness scores of six-week-old (A) male and (B) female mice treated with saline or LPS and exposed to either milk control (Saline + Milk, LPS + Milk) or kefir (Saline + Kefir, LPS + Kefir) as a function of time. Data represented as mean ($\pm$ SEM), $n = 10/\text{group}$. The asterisks (*) denote significant difference between probiotic treatments ($p < 0.05$).

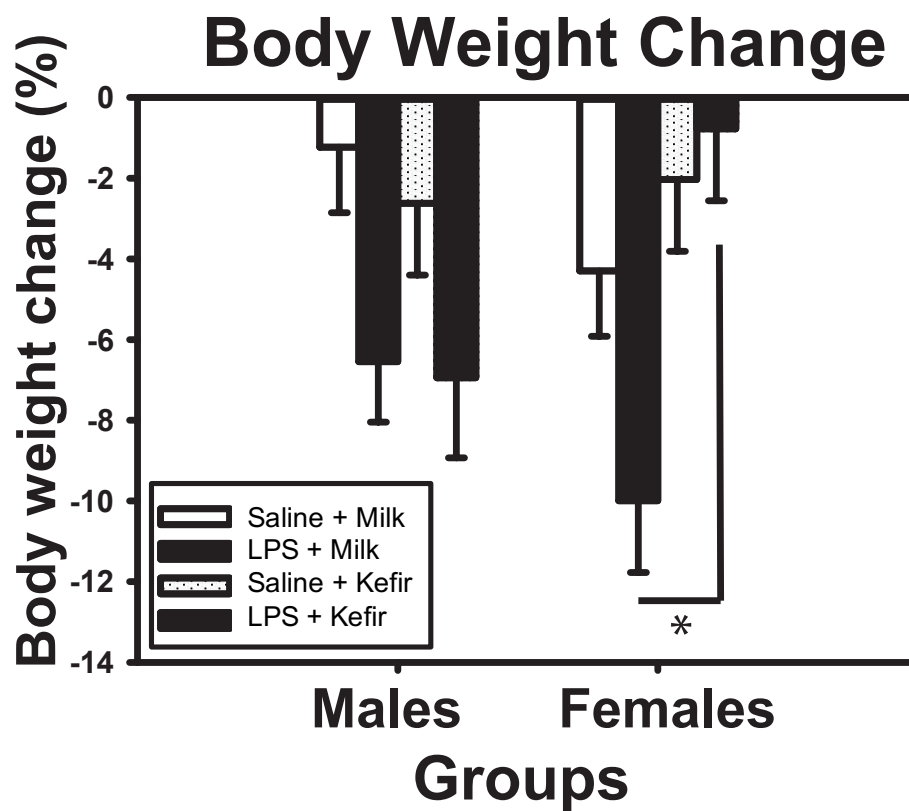


Figure 3. Acute percent body weight change of male and female mice at 48 hours following saline or LPS treatment and exposed to either milk control (Saline + Milk, LPS + Milk) or kefir (Saline + Kefir, LPS + Kefir). Data represented as mean ($\pm$ SEM); * $p < 0.05$, $n = 10$ /group.

		Serum Cytokine Concentration (pg/mL)				
		IL-6	IL-10	IL-12	TNF α	IFN γ
Male	Kefir +LPS	8271.22 $\pm$ 703.22	397.00 $\pm$ 49.90	39.69 $\pm$ 18.29	56.93 $\pm$ 15.50	4931.11 $\pm$ 850.37
	Kefir + Saline	3.78 $\pm$ 667.14	1.14 $\pm$ 47.05	1.93 $\pm$ 17.25	2.84 $\pm$ 14.61	1.633 $\pm$ 850.37
	Control + LPS	9918.22 $\pm$ 703.22	574.22 $\pm$ 47.05	139.27 $\pm$ 17.25	116.88 $\pm$ 14.61	6147.11 $\pm$ 850.37
	Control + Saline	24.88 $\pm$ 745.88	5.07 $\pm$ 53.35	1.85 $\pm$ 18.29	8.52 $\pm$ 15.50	10.85 $\pm$ 901.95
Female	Kefir + LPS	5508.51 $\pm$ 667.14	303.59 $\pm$ 47.05	56.64 $\pm$ 16.37	67.01 $\pm$ 14.61	5910.44 $\pm$ 806.73
	Kefir + Saline	2.71 $\pm$ 703.22	1.09 $\pm$ 47.05	1.58 $\pm$ 17.25	2.08 $\pm$ 14.61	.36 $\pm$ 850.37
	Control + LPS	5386.83 $\pm$ 861.27	345.17 $\pm$	41.68 $\pm$ 21.13	81.64 $\pm$ 17.90	5822.17 $\pm$ 901.95
	Control + Saline	.42 $\pm$ 703.22	.45 $\pm$ 47.05	.88 $\pm$ 17.25	.40 $\pm$ 14.61	.16 $\pm$ 850.37

Data are expressed as means $\pm$ SEM.

Table 2. IL-6, IL-10, IL-12, TNF α and IFN γ serum concentration after LPS treatment or sterile saline in kefir and milk control groups.

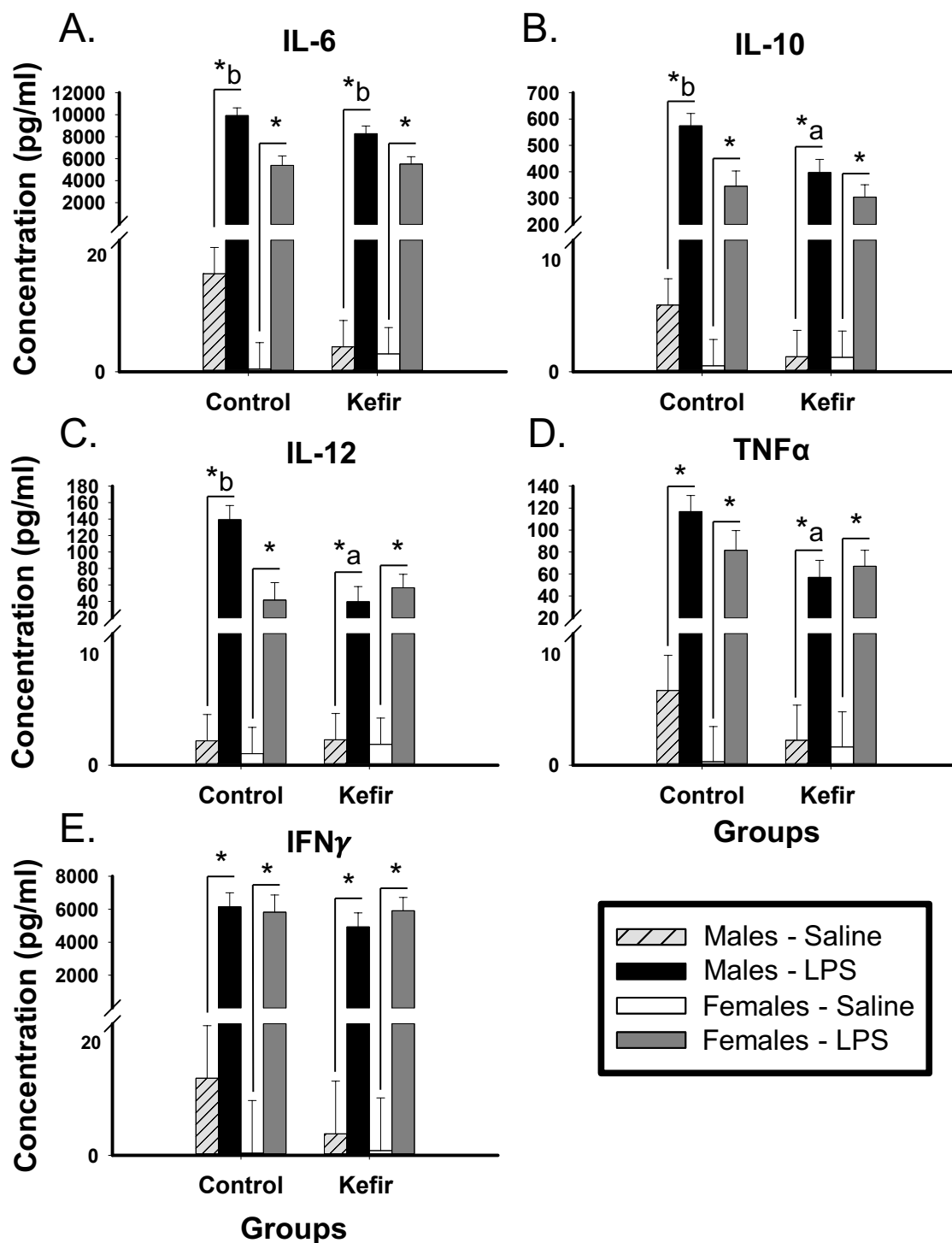


Figure 4. Pro- and anti-inflammatory serum cytokine concentrations (pg/mL) from blood in male and female mice treated with saline or LPS and following 1 week of either milk control or probiotic treatment. Data represented as mean ($\pm$ SEM); * $p < 0.05$, 'a' denotes a significant difference ($p < 0.05$) between the probiotic and milk control treatment, 'b' denotes a significant difference between males and females, $n = 6/\text{group}$.

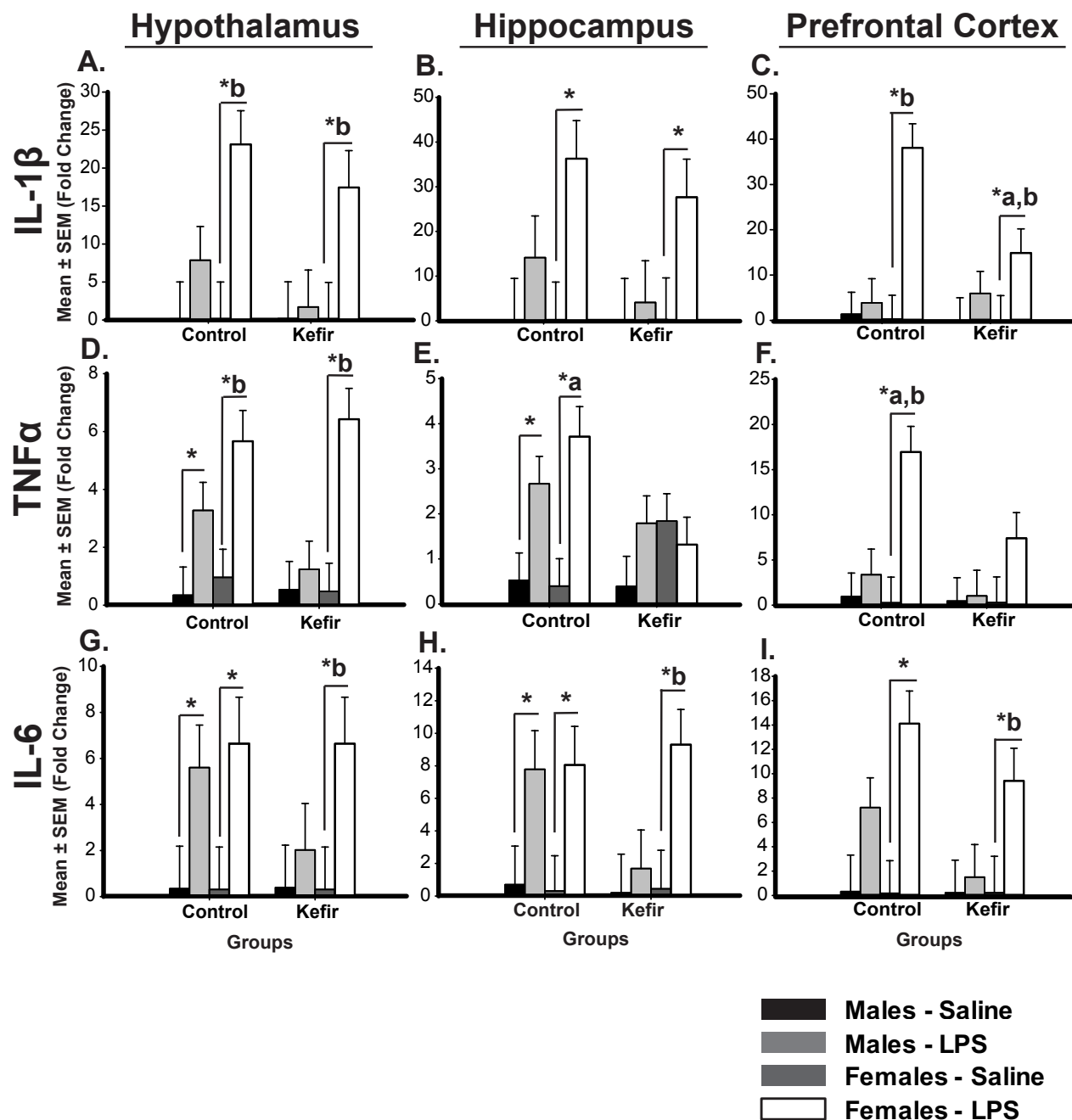


Figure 5. mRNA cytokine expression (fold change) of IL-6, IL-1 β and TNF α mRNA in the hypothalamus, hippocampus and prefrontal cortex (PFC) in male and female mice treated with saline or LPS and following one week of probiotic treatment (milk control or kefir). Data represented as mean ($\pm$ SEM); * p < 0.05, 'a' denotes a significant difference (p < 0.05) between the probiotic and milk control treatment, 'b' denotes a significant difference between males and females, n = 6/group.

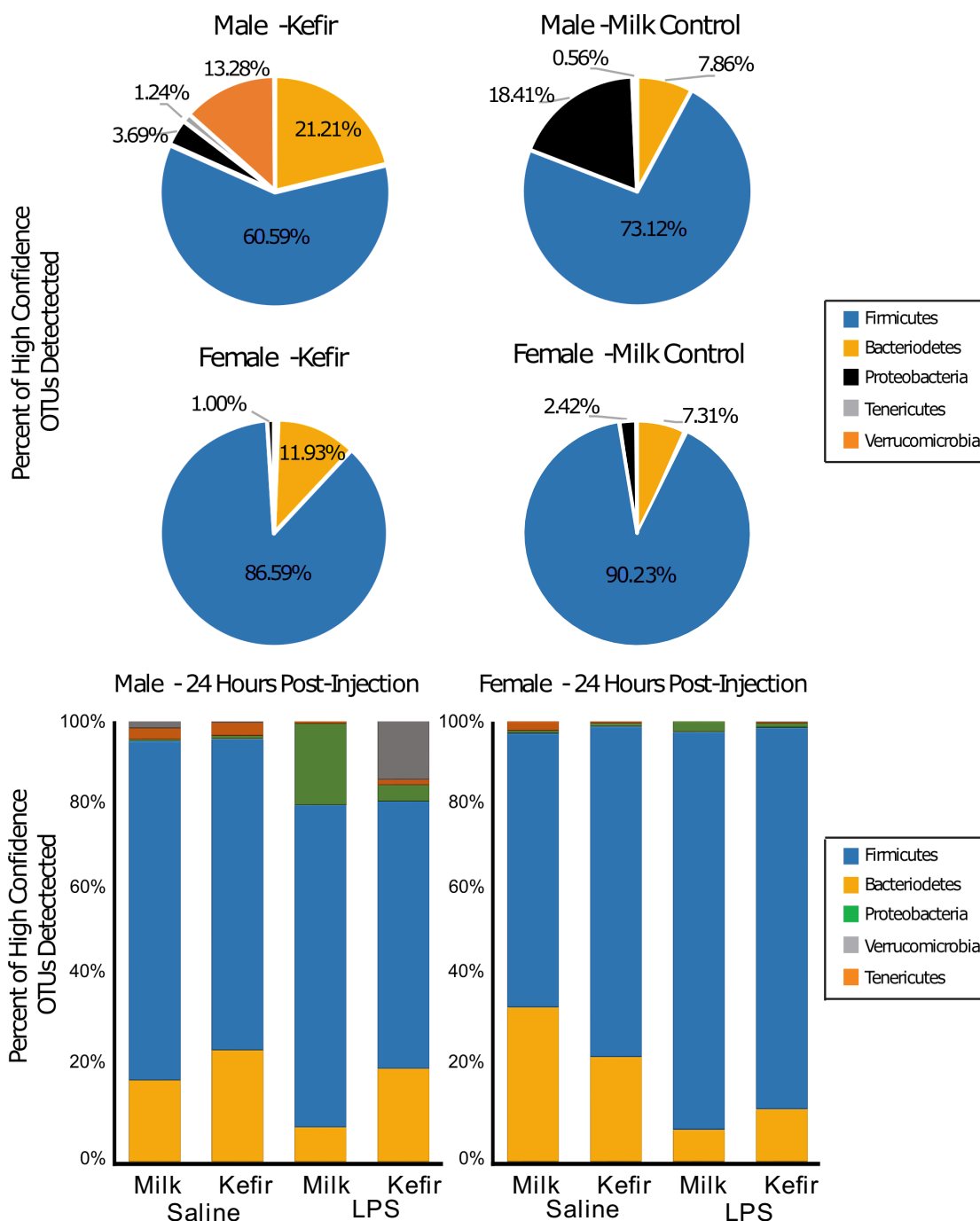


Figure 6. Relative abundance of phylum at 24 hours post saline or LPS treatment and following 1 week of probiotic treatment (milk control or kefir) in 6-week-old male and female mice; represented as stacked bar graphs. Pie charts represent a comparison of milk control to kefir in male and female LPS-treated mice. Data represented as mean ($\pm$ SEM); $*p < 0.05$, $n = 10$ /group).

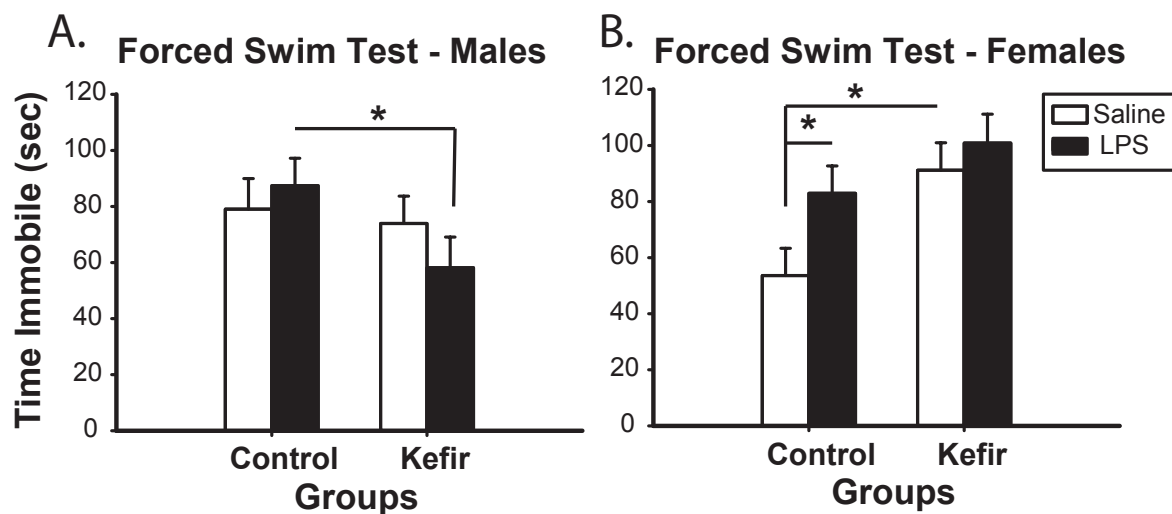


Figure 7. Duration of immobility (sec) over a 5-minute trail of the forced swim test in 10-week-old male and female mice that were previously exposed with a two-week course of probiotic treatment (milk control or kefir) from 5-7 weeks of age and treated with saline or LPS at 6 weeks of age. Data represented as mean ($\pm$ SEM); * $p < 0.05$, $n = 10$ /group.

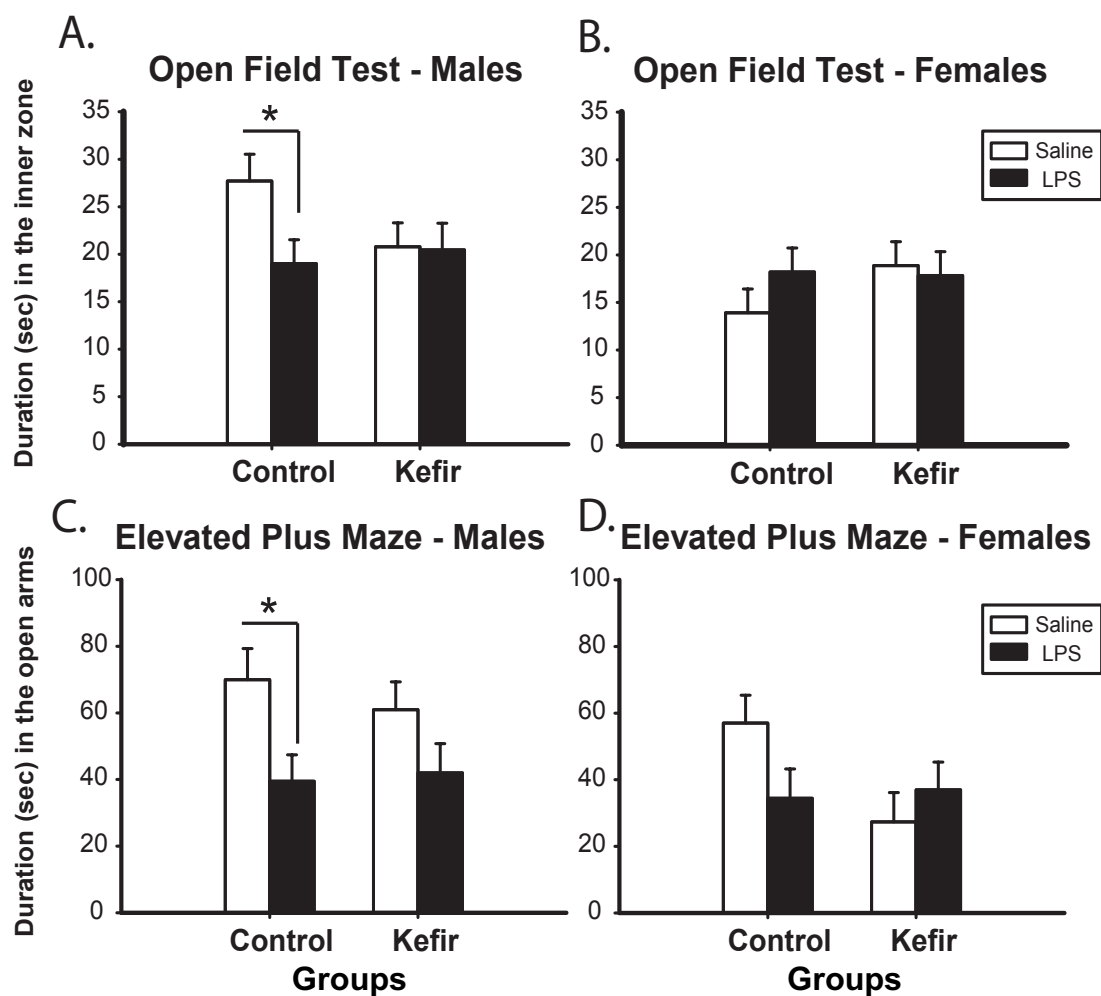


Figure 8. Time spent (sec) in the open arms of the elevated plus maze (EPM) and the inner zone of the open field test (OFT) over 5-minute trials in male and female mice that were previously exposed with a two-week course of probiotic treatment (milk control or kefir) from 5-7 weeks of age and treated with saline or LPS at 6 weeks of age. Data represented as mean ($\pm$ SEM); $*p < 0.05$, $n = 8-10$ /group.

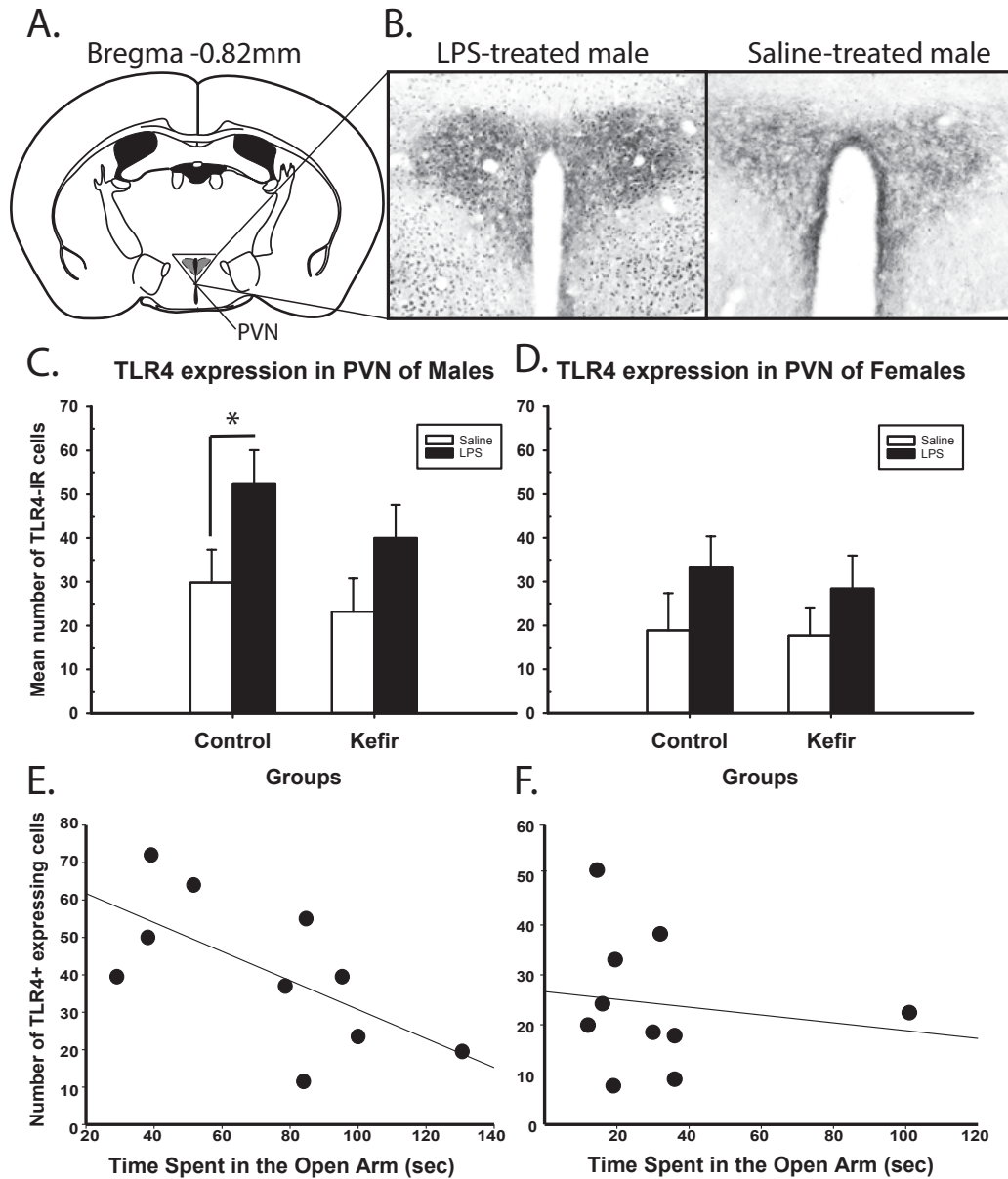
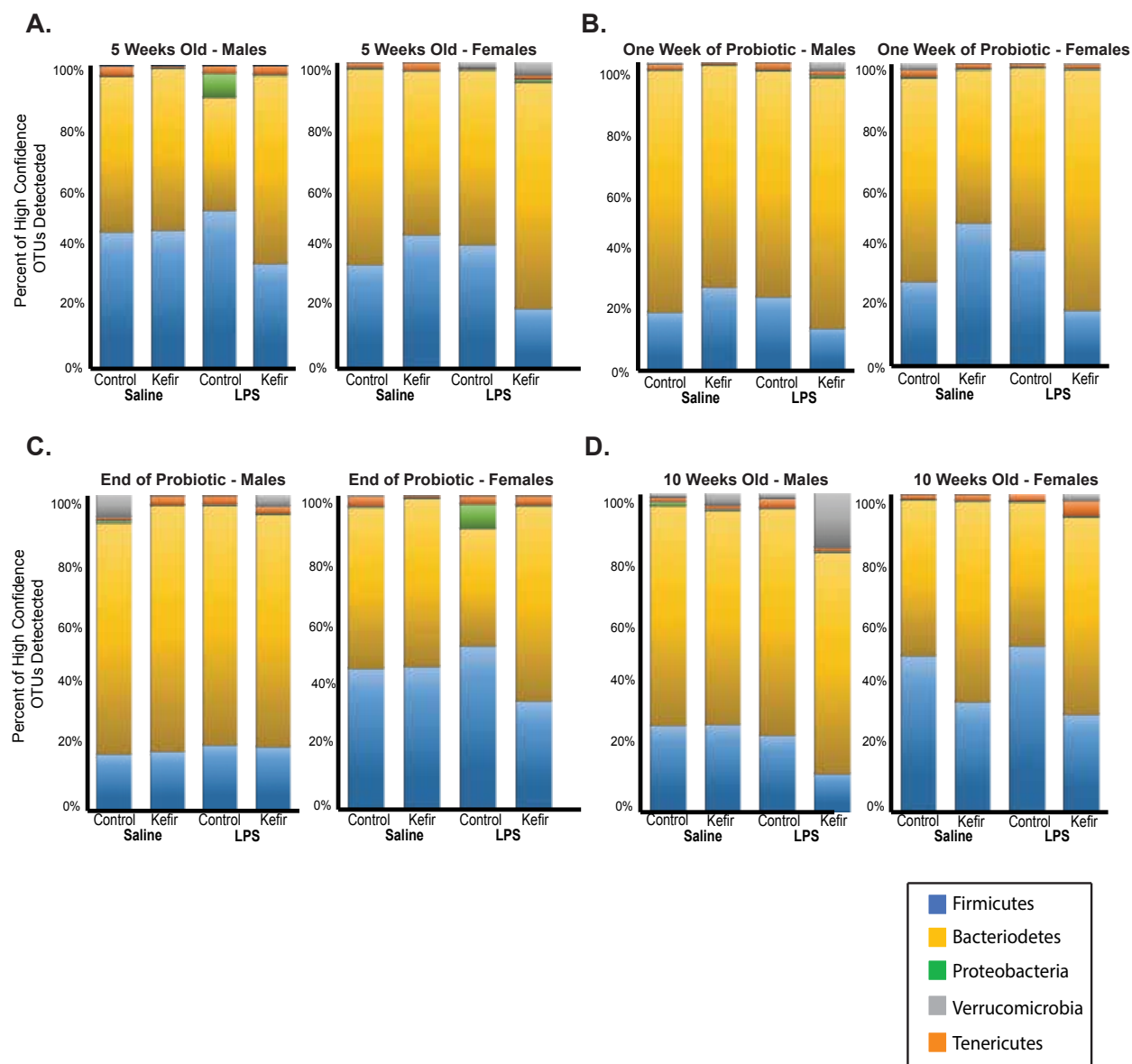
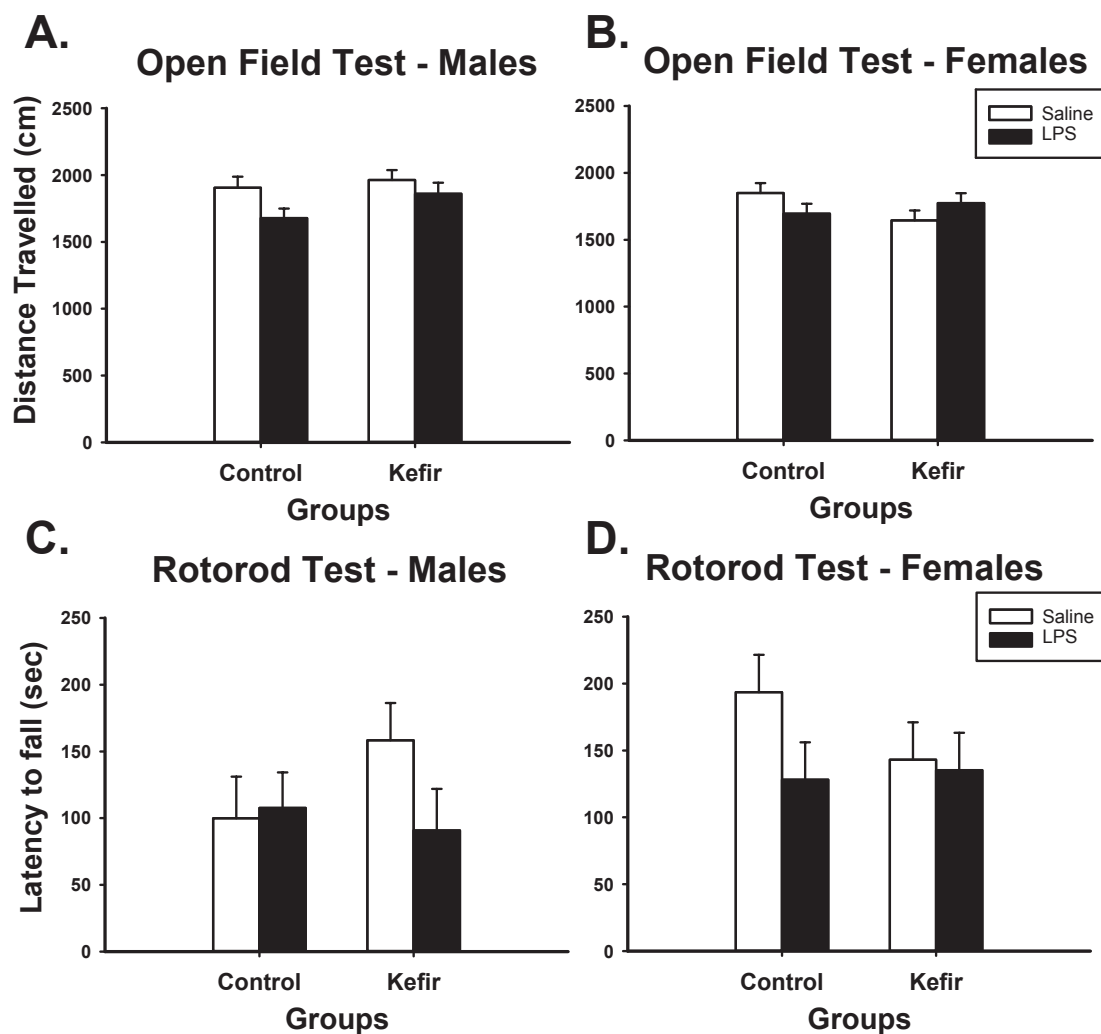


Figure 9. TLR4 immunoreactive cells in the PVN at 30 minutes following termination of a 30-minute restraint stress in adult male and female mice. Plate 38 from the Franklin and Paxinos (1997) mouse brain atlas depicts the paraventricular nucleus of the hypothalamus (PVN) (A). Photomicrographs depicting a comparison of TLR4 expression in LPS-treated male and a saline-treated male in the non-probiotic control condition (B). Bar graphs represent mean number of TLR4 immunoreactive cells in the PVN of adult male and female mice following restraint that were previously exposed to a two-week course of probiotic treatment (milk control or kefir) from 5-7 weeks of age and treated with saline or LPS at 6 weeks of age (C). Scatter plot representing a negative correlation between number of TLR4+ expressing cells and time spent in the open arms of the EPM in adult males (E), but not adult females (F). Data represented as mean ($\pm$ SEM) for TLR4 immunoreactive cells (D,E); * $p < 0.05$, $n = 5-6$ /group.



Supplemental Figure 1. Relative abundance of phylum at time points across the study in male and female mice; represented as stacked bar graphs.



Supplemental Figure 2. Sex affects motor performance yet, no other treatment differences were observed. Distance travelled on the EPM (**A**, **B**) showed saline-injected males moved a greater distance compared to saline-injected females in the kefir probiotic condition ($p < 0.005$). Rotorod test assessed the latency to fall on trial 1 in (**C**) males and (**D**) females. Statistical analyses revealed no significant main effects or interactions. Other mobility tests included zone transitions and travel velocity on the open field test (OFT). In the milk control condition, saline-treated males had more zone transitions compared to saline-treated females ($p = .001$). No other main effects or interactions were observed.

Article 2

Pubertal probiotic blocks LPS-induced anxiety and the associated neurochemical and microbial outcomes, in a sex dependent manner.

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Abstract

Puberty is a critical period of neural development, and exposure to stress and inflammation during this period is thought to increase vulnerability to mental illness. The gut microbiome influences brain functioning and behavior and impacts mental health. Yet, the role of the gut microbiome during puberty, a period during which mental health conditions tend to onset, remains largely uninvestigated. We first examined age and sex differences in gut microbial changes among CD-1 mice exposed to an immune challenge at 6 weeks of age (during the pubertal stress-sensitive period) or at 10 weeks of age (in adulthood) (Experiment 1). Compared to their adult counterparts, pubertal males and females showed more significant changes in gut microbial composition following lipopolysaccharide (LPS) treatment, including the depletion of numerous bacterial genera such as *Lactobacillus*. Given the beneficial effects of *Lactobacillus* strains on stress and behaviour, we next investigated whether replenishment of the gut with the probiotic *Lactobacillus reuteri* (*L. reuteri*) throughout pubertal development would modulate LPS-induced sickness and enduring effects on memory dysfunction, anxiety-like behaviour and stress reactivity in adulthood (Experiment 2). LPS treatment at 6 weeks of age created enduring changes in anxiety-like behaviors among males only. Similarly, only males showed the protective effects of *L. reuteri* supplementation during puberty in preventing longstanding LPS-induced changes in anxiety-like behavior and stress-induced brain activation. These findings demonstrate that colonizing the gut with *L. reuteri* during puberty modulates sickness responses and enduring behavioural and neurochemical outcomes in a sex-specific manner. Therefore, colonizing the gut with beneficial microbes may protect against the development of mental illnesses in adulthood.

Keywords: Adolescence, Gut microbiome, Sex, Immune challenge, Lipopolysaccharide, Stress

1. Introduction

Puberty is a period of sexual maturation that is accompanied by physical changes (Sisk and Foster, 2004) and rapid central nervous system (CNS) reorganizing and remodeling (Levitt, 2003; Schulz et al., 2009). These rapid and complex pubertal changes render the CNS particularly sensitive to stressors (Ge et al., 2001; Grant et al., 2004) like an immune challenge (lipopolysaccharide; LPS) (Olesen et al., 2011; Ismail et al., 2011; Holder & Blaustein, 2014). In mice, a single exposure to the bacterial endotoxin LPS during puberty, at six weeks of age, causes enduring impairments in cognitive functioning, sexual receptivity, and anxiety- and depression-like behaviours during adulthood (Ismail et al., 2011, Olesen et al., 2011; Kolmogorova et al., 2019). These enduring behavioral alterations are limited to LPS treatment at six weeks of age, a period of heightened stress sensitivity during puberty, as LPS treatment at younger or older ages during the peri-adolescent period does not induce enduring impairments in reproductive and non-reproductive behaviors in inbred C57B1/6 or outbred CD1 mice (Laroche et al., 2009; Olesen et al., 2011; Ismail et al., 2011). Recent findings show that there are also important age and sex differences in the acute immune response to LPS (Girard-Joyal et al., 2015; Cai et al., 2016; Sharma et al., 2018). For example, LPS treatment induces greater hypothermia in adult male mice compared to their pubertal counterparts (Cai et al. 2016). Moreover, LPS-treated males display more sickness symptoms and greater hypothermia compared to LPS-treated females (Cai et al., 2016). Further investigation of these age and sex differences in immune response may help elucidate the mechanism involved in the enduring behavioral alterations.

One system suggested to influence the developing brain and immune response is the gut microbiome (Borre et al., 2014; Jašarević et al., 2018). The gut microbiome is a diverse and

dynamic system that contains trillions of microorganisms (Qin et al., 2010). Commonly referred to as “the gut-brain axis”, the gut microbiome and the brain communicate through the autonomic and enteric nervous systems, and neuroimmune and neuroendocrine pathways (Browning and Travagli, 2014; Cryan & Dinan, 2011; Foster et al., 2017 Grenham et al., 2011). These microorganisms play a large role in overall health and are involved in systemic immune regulation, inflammation, and metabolic functions (Fung et al., 2017). The gut microbiome has also been linked to anxiety and cognitive functioning in both humans and rodent models (Kennedy et al., 2016; Cryan & Dinan, 2012). For example, patients with chronic gut disorders such as inflammatory bowel disease (IBS) commonly show psychiatric comorbidities, like anxiety and depression (Kennedy et al., 2016; Cryan & Dinan, 2012). In addition, perturbations to the microbiota via pathogenic agents, like *Citrobacter rodentium*, increases anxiety-like behaviour and stress-induced memory dysfunction (Gareau et al., 2011). In a study by Bercik and colleagues (2011), increased anxiety-like behaviour, induced with dextran sodium sulfate (DSS), was ameliorated with daily probiotic administration of *Bifidobacterium Longum* (Bercik et al., 2011). Probiotics are live microorganisms that are beneficial to overall health (Hill et al., 2014). A number of probiotics, like the *Lactobacillus* genus, have also shown positive effects on anxiety- and depression-like behaviors (Messaoudi et al., 2011; Arseneault-Breard et al., 2012). For example, manipulation of the gut with the probiotic *Lactobacillus rhamnosus* reduced stress-induced corticosterone (CORT) release and reduced GABA_{BLb} mRNA in the hippocampus and amygdala (Bravo et al., 2011).

There is accumulating evidence that probiotic interventions benefit mental health, cognition, and other neurochemical and behavioural outcomes (Arseneault-Breard et al., 2012; Bercik et al., 2011; Bravo et al., 2011; Messaoudi et al., 2011); however, the mechanisms have

not been fully elucidated. Moreover, the effects of specific species of beneficial bacteria during puberty remain largely uninvestigated. Therefore, we investigated age and sex differences in LPS-induced changes in gut microbial composition (Experiment 1). We then examined how colonizing the gut with the probiotic *Lactobacillus reuteri* (*L. reuteri*) throughout pubertal development affects LPS-induced changes in sickness behavior and body weight change and enduring effects on cognitive-functioning, anxiety-like behavior and cellular activation in the hippocampus following a novel stressor in adulthood (Experiment 2). We hypothesized that LPS exposure would cause a depletion of multiple bacterial genera and that pubertal mice would show greater LPS-induced microbial changes. We also hypothesized that a two-week treatment of *L. reuteri*, beginning one week before LPS treatment would protect against LPS-induced sickness and prevent enduring negative outcomes (i.e., anxiety-like behaviour, and spatial memory and c-Fos expression in the hippocampus following a novel stressor) in adulthood.

2. Material and methods

2.1. Animals

Sixty male and 60 female CD-1 mice were shipped at three weeks of age from Charles River Laboratories. All mice were sex-segregated and housed in controlled temperature ($24\pm 2^{\circ}\text{C}$) rooms on a reversed light:dark cycle (14h:10h; lights off at 9:00am). Mice were pair-housed in polycarbonate Lexan cages (17 x 28 x 12 cm [width x length x height]) with *ad libitum* access to food (Harlan Laboratories, Inc., Madison, WI, USA, T2018–Global 18% rodent) and water. Cages were bedded with Teklad Corn Cob (Harlan Laboratories, Inc., Madison, WI, USA) and enriched with a square piece of Nestlet (Ancare Corp., Bellmore, NY, USA) and a refuge cardboard hut (Ketchum Manufacturing, Inc., Brockville, ON, CA, USA). All mice received equal handling from male and female researchers. Handling occurred during cage changes

(weekly), body weight measures, and behaviour testing. All experimental procedures were approved by the Animal Care Committee of the University of Ottawa.

2.2. Experimental procedures

2.2.1. Experiment 1: Effect of LPS treatment on microbial composition in pubertal and adult male and female mice.

At six (pubertal group) or 10 weeks (adult group) of age, female (n = 40) and male (n = 40) CD1 mice received an injection of either sterile saline or LPS (1.5mg/kg). Fecal samples were collected 24 hours, and 8 weeks following treatment. Microbial composition was analyzed using 16S rRNA sequencing.

*2.2.2. Experiment 2: Effect of *L.reuteri* on LPS-induced sickness responses and enduring effects on memory, anxiety-like behavior, and stress reactivity in adulthood.*

A second cohort of males (n = 20) and females (n = 20) was used to examine the effects of the probiotic *L.reuteri* on acute LPS-induced sickness behaviour and long-term memory dysfunction, anxiety-like behavior and stress reactivity in adulthood. These mice were exposed to either a probiotic solution of *L.reuteri* (5×10^7 CFU/mL) or a control broth in their drinking bottle, beginning at five weeks of age for the following 14 days. The solution was replaced daily. At six weeks of age, mice received an IP injection of either sterile saline or LPS (1.5 mg/kg). At 10 weeks of age, they were subjected to a series of behaviour tasks to examine anxiety, spatial memory and stress responses. All mice were tested during the dark phase of their light-dark cycle.

2.3. Pubertal Immune Stress

2.3.1. Lipopolysaccharide (LPS)

Lyophilized LPS (obtained from *Escherichia coli* serotype O26:B6; #L3755; Sigma Aldrich Canada, Oakville, ON) was dissolved in 0.9% (w/v) sterile aqueous sodium chloride (saline) solution (Ricca Chemical Company; #R7210000-4A7210-1). All mice were injected intraperitoneally at either 6 weeks (pubertal stress-sensitive period) or 10 weeks of age (in adulthood) with either LPS (1.5mg/kg) or an equivalent volume of 0.9% sterile saline. This LPS dose elicits mild sickness for 48 hours in both sexes (Cai et al., 2016). Under our housing conditions, 6-week-old CD1 female mice are not displaying estrous cycling (Ismail and Blaustein, unpublished observation) and male scrotal size has not yet reached adult size (Lamba, Murray & Ismail, unpublished observations).

2.3.2. Sickness Monitoring

Behavioral signs of sickness were assessed at 2, 4, 8, 12, 24, and 48 hours following injection by two observers blind to treatment conditions (as described in Kolmogorova, Murray, Ismail, 2017). Mice received a score between 0 and 4 to reflect the number of sickness symptoms (lethargy, huddling, ptosis, and piloerection) displayed at each time point. Body weights (g) were recorded prior to saline or LPS injection (i.e., baseline) and at 48 hours after injection. Percentage changes in body weight from baseline were calculated as described in Kolmogorova and colleagues (2017).

2.4. Gut microbiome analyses and treatment

2.4.1. Fecal Sample Collection

Fecal samples were collected at 24 hours following saline or LPS injection to examine acute age and sex differences on gut microbiota. A second collection was conducted 8 weeks post-treatment, in adulthood, to elucidate enduring effects of LPS on gut microbial composition. To collect fecal samples, mice were briefly removed from their cage and defecation was induced

by applying gentle pressure to their back. Samples were immediately frozen using liquid nitrogen, placed in Eppendorf tubes, and stored at -80°C until DNA extraction. Fecal samples from pair-housed mice were combined and analyzed as one sample for DNA extraction ($n = 3\text{--}4$ homogenized sample/group).

2.4.2. DNA Extraction from Fecal Samples & bTEFAP Sequencing

DNA extraction was performed using a PowerSoil DNA Isolation Kit (MO BIO Laboratory Inc.) according to the manufacturer's recommendations. Fecal samples were thawed and fecal pellets were homogenized by vortexing for 3min. One mL aliquots of each sample were centrifuged at 13,200rpm (16,100rcf) for 10min at room temperature. The supernatant was discarded, and the remaining pellet was resuspended in 400 μL of nuclease-free water. DNA concentration and purity were evaluated by optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at wavelengths of 230, 260, and 280nm and revealed a reading ratio between 1.6-3.2. Extracted DNA samples were sent to the Research and Testing Laboratory (Lubbock, Texas) where 16S-based tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed for the identification of bacterial diversity (see Supplemental Material for more details). The relative abundance of microbial taxa ('operational taxonomic units' OTUs; Blaxter et al., 2005) in each sample was analyzed at both the phylum and genus levels.

2.4.3. Probiotic preparation

L. reuteri (ATCC[®] laboratory, 23272[™]) was grown on an agar preparation. The *L. reuteri* probiotic formula was prepared by adding 10 μL of saturated bacteria culture into 500mL of autoclaved Lactobacilli Broth AOAC. The liquid culture was incubated at 37°C for 24 hours. Once the incubation period was completed, 25mL of the liquid culture was placed into tubes with

25mL of glycerol and frozen at -80°C until use. Four tubes of probiotic were diluted into 800ml of distilled water and 100mL was placed into each drinking bottle. The final concentration of *L. reuteri* in drinking water was 5×10^7 CFU/mL (see Supplemental Material for more details). Mice in the non-probiotic condition were exposed to a control broth that was prepared in the same manner as the probiotic formula but without the *L. reuteri* bacteria. The probiotic formula or the control broth bottles were weighed and replenished daily. There was no difference in the average consumption between the groups.

2.5. Behavioral Testing

Behaviour testing was carried out over a two-week period with a 48-hour rest period between each test (OFT, MWM, Restraint Stress) to avoid any carry-over effects. Mice were exposed to all of the behavior tests in a consistent order, but the order of testing was varied between mice such that females were tested first on the first day and males were tested first on the next day. Testing was carried out between 12p.m.-3p.m. during the dark phase of their cycle.

2.5.1. Open Field Test (OFT)

Anxiety-like behavior and locomotion were assessed using the OFT (Carola et al. 2002). Mice were placed in a square arena (100cm X 100cm) for five minutes. The distance (cm) travelled and time spent in the inner (30cm X 30cm) and outer (20cm from each wall) portions of the open field were recorded using an overhead closed-circuit Panasonic video system and analyzed using the Noldus Ethovision software. Greater duration in the inner portion of the field is an indicator of reduced anxiety-like behaviour, while more time spent in the outer portion of the field indicates greater anxiety-like behaviour (Prut and Belzung, 2003). The distance travelled was examined as a measure of locomotor activity.

2.5.2. Morris Water Maze (MWM)

Spatial learning and memory were assessed using the Morris water-maze (MWM) (Vorhees & Williams, 2006). A circular pool (1.7 m diameter with a rim 50 cm high) was filled with water (30 cm; $23\pm 1^{\circ}\text{C}$) made opaque by non-toxic white tempera paint (Scholar's Choice Inc.). Spatial cues were placed on the walls of the maze. The maze was divided into north, south, east and west quadrants. An escape platform was placed 1 cm below the surface of the water in the north (target) quadrant. The acquisition phase (days 1-4) consisted of four 60-second trials with 30-second inter-trial intervals with a 24-hour rest between each day. Mice were semi-randomly placed in a non-target quadrant and were given 60 seconds to find the platform. Latency to reach the platform was recorded during each trial. Spatial retention was assessed during the probe phase (day 5) and consisted in placing the mouse at a novel start position in the maze for a single 60-second trial during which the hidden platform was removed. Total distance (cm) travelled in the arena and in the target quadrants, duration (s) in the target and non-target quadrants, and average distance (cm) to the platform in each trial were recorded by an overhead closed-circuit Panasonic video system and analyzed with Noldus EthoVision[®] XT software.

2.6. Restraint Stress

Forty-eight hours after the MWM, mice were exposed to a restraint stress for 30min and euthanized one hour following the end of the restraint.

2.6.1. Brain extraction

Mice received an intraperitoneal injection of Euthanyl (prepared from Euthansol; Merck Animal Intervet Canada Corp; Kirkland, Quebec) one hour following the end of restraint. Brains were extracted and flash frozen in liquid nitrogen. Brain tissue was stored at -80°C until they were sectioned. Frozen brains were sliced using a cryostat into coronal sections (15 μm thickness) and mounted directly onto Superfrost plus slides (Fisherbrand). Slides were stored at -80°C until

immunocytochemistry.

2.6.2. Immunocytochemistry

Immunocytochemistry was used to examine c-Fos expression. Slide-mounted sections were fixed overnight in 4% paraformaldehyde. Sections were rinsed in tris-buffered saline (TBS, pH 7.6) and incubated in a solution of 1.1763g sodium citrate in 80mL of TBS. To quench unreacted aldehyde groups, the sections were rinsed with TBS and incubated for 30 minutes in 0.6003g of glycine in 80mL of TBS. Following additional TBS rinses, the sections were incubated for 30 minutes in a concentrated blocking solution containing TBS, 20% normal goat serum, 0.3% Triton-X, and 1% hydrogen peroxide and then incubated overnight at room temperature in a solution containing a primary antiserum specific to c-Fos (Ab-5 rabbit anti c-Fos; PC05 Millipore; 1:10), validated for use in frozen tissue (D'Agostino et al., 2016). After washing in a diluted blocking solution (1% NGS, 0.02% Triton-X in TBS), sections were incubated for 60 min in the secondary antibody (biotinylated goat Anti-rabbit IgG; BA-1000; Vector Labs; 1:500) solution in TBS, followed by a one-hour incubation in the Vectastain ABC system (Vector Vectastain ABC kit Elite Pk-6100 standard; Vector Laboratories, Burlingame, CA, USA). The sections were again washed with TBS. Then, the sections were incubated in freshly prepared diaminobenzidine (DAB) solution (DAB Peroxidase Substrate kit, SK-4100 Vector Laboratories, Burlingame, CA, USA), followed by rinses in TBS. Slides were coverslipped using Permount (Fisher Scientific, Permount Adhesive, SP15-500).

2.6.3. Image Analysis and Cell Quantification

The cornu ammonis (CA)-1, CA3, and the dentate gyrus (DG) of the hippocampus (i.e., bregma: -2.18mm, Franklin and Paxinos, 1997) were examined for c-Fos expression based on one representative section for each mouse. Images were captured at 20X on an Olympus BX51

light microscope connected to a Jenoptik ProRes MF scan camera and quantified using unbiased counting on ImageJ software (version 1.48) by two raters blind to treatment conditions. Adjusted selections for the general shape of the regions of interest were built with ImageJ and the same selection was used to analyze the specific brain region (i.e. CA1, CA3 or DG) across all mice. Thresholding was applied to each image to dissociate c-Fos-stained cells from background. Parameters related to the circularity (0.1-0.9) and size of cells (50-1500 pixels²) were set and used across all mice.

2.7. Statistical analyses

All statistical analyses were performed using the SPSS V22 software. All data were initially screened for outliers. Data that was 1.96 SD away from the mean were considered outliers (Tabachnick & Fidell, 2007). One outlier was removed from the MWM analysis on the latency to reach platform (Day 2). Relative abundance of bacteria at the genus and phylum levels were performed using three-way between-subject analyses of variance (ANOVA) to examine the effect of age (6 or 10 weeks old), treatment (saline or LPS), sex (male or female) at 24 hours post-injection. A two-way between-subject ANOVA was conducted to examine the enduring effect of LPS treatment and sex on the relative abundance of phyla at 8-weeks post-treatment.

A four-way mixed ANOVA was used to examine the within-subject effect of time (2, 4, 8, 12, 24 and 48 hours), and the between-subject effects of sex, age, probiotic treatment (control broth or probiotic *L.reuteri*) and immune challenge (saline or LPS) on sickness responses (as published in Cai et al., 2016; Girard-Joyal & Ismail, 2017; Kolmogorova et al., 2019). A three-way between-subject ANOVA was used to examine the influences of sex, probiotic treatment, and immune challenge on the behavioural measures, and c-Fos-expression in the CA1, CA3 and DG regions of the hippocampus. Significant interactions were followed by pairwise comparisons

and a Bonferroni correction factor was applied to control for the number of comparisons. The criterion for statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Experiment 1: Effect of LPS treatment on microbial composition in pubertal and adult male and female mice.

3.1.1 LPS-induced changes in the gut microbiota

LPS treatment significantly reduced percentage of Bacteroidetes ($F_{(1,17)} = 17.29, p = .001, \eta^2_p = .504$), Firmicutes ($F_{(1,17)} = 12.423, p = .003, \eta^2_p = .422$), Candidatus Saccharibacteria ($F_{(1,17)} = 7.344, p = .015, \eta^2_p = .302$) and Cyanobacteria ($F_{(1,17)} = 4.45, p = .05, \eta^2_p = .207$), and increased Deferribacteres ($F_{(1,17)} = 14.54, p = .001, \eta^2_p = .461$), Proteobacteria ($F_{(1,17)} = 4.46, p = .05, \eta^2_p = .208$) and Verrucomicrobia ($F_{(1,17)} = 24.473, p < .001, \eta^2_p = .590$) in all mice regardless of age and sex. Aging significantly reduced percentage of Bacteroidetes ($F_{(1,17)} = 22.118, p < .001, \eta^2_p = .565$) and Candidatus Saccharibacteria ($F_{(1,17)} = 6.228, p = .023, \eta^2_p = .268$). There was a significant age x LPS interaction on the percentage of Bacteroidetes ($F_{(1,17)} = 33.42, p < .001, \eta^2_p = .663$) and Candidatus Saccharibacteria ($F_{(1,17)} = 6.48, p = .021, \eta^2_p = .276$) and a significant LPS x sex interaction on Chordata ($F_{(1,17)} = 4.95, p = .04, \eta^2_p = .226$) at 24 hours following treatment (Fig 1; note: phyla that are expressed at <10% in the gut microbiome are represented in the “Other” category (e.g. Candidatus Saccharibacteria, Cyanobacteria, and Deferribacteres).

Saline-injected adult males displayed reduced abundance of Bacteroidetes (mean difference; $MD = 21.641$, standard error; $SE = 4.558, p < .001$), Actinobacteria ($MD = 1.049, SE = .368, p = .011$) and Candidatus Sacchribacteria ($MD = 1.166, SE = .520, p = .039$) phyla compared to their pubertal male counterparts (Fig. 1). Similarly, adult female mice displayed

significantly less Bacteroidetes ($MD = 21.103$, $SE = 3.814$, $p < .001$), Cyanobacteria ($MD = .951$, $SE = .418$, $p = .036$) and Candidatus Saccharibacteria ($MD = 1.180$, $SE = .435$, $p = .015$) phyla and significantly greater Firmicutes phylum ($MD = 18.631$, $SE = 8.572$, $p = .044$) compared to their pubertal female counterparts.

Pubertal male and female mice injected with LPS showed significantly greater abundance of Verrucomicrobia phylum ($MD = 33.578$, $SE = 8.157$, $p = .001$; $MD = 22.123$, $SE = 7.064$, $p = .006$) and significantly reduced abundance of Bacteroidetes ($MD = -21.398$, $SE = 4.08$, $p < .001$; $MD = -19.122$, $SE = 3.531$, $p < .001$) and Candidatus Saccharibacteria ($MD = -1.273$, $SE = .465$, $p = .014$; $MD = -1.173$, $SE = .403$, $p = .01$) phyla compared to saline-injected controls. LPS-treated pubertal males also showed significantly greater abundance of Deferribacteres ($MD = .785$, $SE = .316$, $p = .023$) and less Actinobacteria ($MD = -1.086$, $SE = .329$, $p = .004$) phyla compared to saline controls. LPS-treated adult males showed significantly less abundance of Firmicutes phylum ($MD = -22.314$, $SE = 10.245$, $p = .044$) and more Deferribacteres phylum ($MD = .919$, $SE = .353$, $p = .018$) compared to saline controls. LPS-treated adult females showed significantly less Firmicutes ($MD = -25.373$, $SE = 9.163$, $p = .013$) and Tenericutes ($MD = -.137$, $SE = .054$, $p = .022$) phyla and significantly more Proteobacteria phylum ($MD = 12.125$, $SE = 5.038$, $p = .028$) compared to their saline-injected controls (Fig. 1). No sex differences in LPS-induced changes in phyla abundance were observed.

An analysis was also performed on the bacterial genera shared by all samples (Fig. 2). A total of 62 bacterial genera were shared by all mice in the study. At 24 hours post-injection, both LPS-treated pubertal male and female mice displayed significantly less *Lactobacillus*, *Odoribacter*, *Tannerella*, *Gloeobacter*, *Candidatus Saccharimonas*, *Barnesiella*, *Prevotella* and *Porphyromonas* genera and significantly more *Parabacteroides* and *Akkermansia* genera

compared to saline controls ($p < 0.05$). Pubertal LPS-treated females also displayed significantly less *Bacillus* and *Intestinimoas* genera compared to their saline counterparts ($p < 0.05$). Pubertal LPS-treated males displayed significantly less *Peptoclostridium*, *Lachnospira*, *Robinsoniella*, and *Adlercreutzia* genera compared to their saline counterparts ($p < 0.05$). LPS-treated adult males and females, displayed a significant decrease in *Hespellia*, *Catabacter*, *Adlercreutzia*, and *Anearostipes* genera compared to saline controls ($p < 0.05$). Fewer number of genera were reduced by LPS treatment in adult mice compared to pubertal mice. To examine whether these effects of pubertal immune challenge on gut microbial composition were long-lasting, we examined microbial composition from our pubertal groups again in adulthood. At this time point, no enduring effects of LPS treatment or sex differences were observed ($p > 0.05$).

3.2. Experiment 2: Effect of *L.reuteri* on LPS-induced sickness responses and enduring effects on memory, anxiety-like behavior, and stress reactivity in adulthood.

3.2.1 Sickness behaviour and body weight changes

As expected, LPS treatment induced more sickness behaviors than saline-treated controls ($F_{(1, 32)} = 1628.82, p < .001, \eta^2_p = .981$) and caused more sickness behavior among males compared to females ($F_{(1, 32)} = 24.102, p < .001, \eta^2_p = .430$). The expression of sickness behavior changed over time ($F_{(5, 160)} = 79.11, p < .001, \eta^2_p = .712$) and there was a significant time x sex x LPS treatment interaction ($F_{(5, 160)} = 2.89, p < .05, \eta^2_p = .083$), such that LPS-injected males showed more sickness behaviour at 8, 12, 24, and 48 hours ($p < 0.05$) compared to LPS-injected females (Fig. 3a and b). LPS-injected male mice treated with the probiotic *L. reuteri* displayed less sickness behaviour at 24 hours ($MD = -0.6, SE = 0.258, p = .027$) compared to LPS-treated male exposed to the control broth. In contrast, LPS-treated female mice that received *L. reuteri* showed less sickness behaviour at 4 hours ($MD = -.45, SE = 0.22, p = .05$), but greater sickness

behaviour at 24 ($MD = 1.050$, $SE = 0.258$, $p < .001$) and 48 hours ($MD = .375$, $SE = 0.076$, $p < .001$) compared to LPS-treated females exposed to the control broth.

There was a significant sex x LPS treatment interaction on body weight change at 48 hours post-LPS treatment ($F_{(1, 32)} = 5.78$, $p = .022$, $\eta^2_p = 0.153$). LPS-treated males lost more weight than saline-treated controls, regardless of probiotic treatment ($MD = -8.944$, $SE = 2.113$, $p < .001$; $MD = -7.235$, $SE = 2.113$, $p = .002$, respectively) (Fig. 3c). There was no difference in body weight change between mice exposed to *L. reuteri* and controls. All mice gained weight as they aged into adulthood. No enduring effect of LPS ($F_{(1, 32)} = 0.042$, $p = .839$, $\eta^2_p = 0.001$) or probiotic treatment ($F_{(1, 32)} = 0.806$, $p = .376$, $\eta^2_p = 0.025$) were observed on body weight in adulthood.

3.2.2. Morris Water Maze (MWM)

A significant main effect of testing day ($F_{(3, 87)} = 13.54$, $p < .001$, $\eta^2_p = 0.318$) was found on latency to reach platform. Spatial learning occurred in all mice and the latency to find the platform improved each day in all mice (Fig. 4a and b). There was no effect of sex ($F_{(1, 32)} = 0.96$, $p = .334$, $\eta^2_p = 0.029$), pubertal exposure to probiotic ($F_{(1, 32)} = 0.416$, $p = .524$, $\eta^2_p = 0.013$) or immune challenge ($F_{(1, 32)} = 0.040$, $p = .843$, $\eta^2_p = 0.001$) on performance in the MWM, suggesting that all mice learned at the same relative rate regardless of treatment. During the probe phase (day 5), there was no difference in time spent in the target quadrant based on sex ($F_{(1, 29)} = 0.927$, $p = .344$, $\eta^2_p = 0.031$), pubertal exposure to probiotic ($F_{(1, 29)} = 0.874$, $p = .358$, $\eta^2_p = 0.029$) or immune challenge ($F_{(1, 29)} = 0.047$, $p = .829$, $\eta^2_p = 0.002$) (Fig 4c and d). Probiotic treatment, sex or immune challenge did not affect distance travelled, travel velocity and time spent mobile vs immobile ($p > 0.05$).

3.2.3. Open Field Test (OFT)

A significant sex x probiotic treatment x LPS treatment interaction was found on time spent in the inner zone ($F_{(1,32)} = 4.098, p = .05, \eta^2_p = 0.114$). LPS-treated males exposed to the control broth spent significantly less time in the inner zone ($MD = -7.81, SE = 3.42, p = .029$) compared to their saline-treated counterparts (Fig. 5a). However, there was no difference in time spent in the inner zone between LPS- and saline-treated mice exposed to *L. reuteri* ($p > 0.05$). Saline-treated males exposed to the control broth spent significantly more time in the inner zone compared to their female counterparts ($MD = 10.02, SE = 3.42, p = .006$) (Fig. 5a and b). Saline-treated females exposed to the probiotic spent significantly more time in the inner zone ($MD = 7.16, SE = 3.42, p = .044$) compared to non-probiotic-treated females (Fig. 5b). There was no difference in travel velocity and in distance travelled between treatment groups ($p > 0.05$; Fig. 5c and d).

3.2.4. Cellular activation in the hippocampus

There was a significant sex x probiotic x LPS treatment interaction on c-Fos expression in the CA1 region of the hippocampus ($F_{(1, 28)} = 4.478, p = 0.043, \eta^2_p = 0.138$). Pubertal exposure to *L. reuteri* significantly reduced stress-induced c-Fos expression in the CA1 ($MD = 417.95, SE = 190.208, p = 0.036$) and CA3 regions ($MD = 719.125, SE = 305.855, p = 0.026$) in LPS-treated males compared to their non-probiotic controls (Fig 6a and c). LPS-treated females exposed to *L. reuteri* displayed greater c-Fos expression compared to male counterparts in the CA1 region ($MD = 406.638, SE = 190.208, p = 0.041$). Moreover, in saline-treated females, those that consumed probiotic showed less c-Fos expression in the dentate gyrus of the hippocampus compared to broth-fed controls ($MD = 211.75, SE = 95.708, p = 0.035$) (Fig 6f).

4. Discussion

The pubertal brain undergoes significant reorganizing and remodeling and is highly

vulnerable to stress and inflammation (Ismail et al., 2011, Olesen et al., 2011). Pubertal exposure to LPS has enduring effects on brain chemistry and behaviour into adulthood (Ismail et al., 2011, Olesen et al., 2011; Kolmogorova et al., 2019). Given the limited understanding of the influence of the gut microbiome on pubertal development, we investigated age and sex differences in microbial composition following LPS treatment male and female mice. While we did not collect fecal samples before saline and LPS and thus, were not able to conduct a pre-post-analysis, we believe that the gut microbiome of saline-injected mice is representative of baseline microbiota; however, minor differences after saline-injection are possible. Regardless of sex, pubertal mice had a greater relative abundance of Bacteroidetes compared to their adult counterparts. In contrast, adults had a greater relative abundance of Firmicutes phylum compared to their pubertal counterparts. Firmicutes and Bacteroidetes are the two dominant phyla (Mariat et al., 2009). The ratio of Firmicutes to Bacteroidetes has been implicated in a number of disease states. For example, the increased abundance of Firmicutes and decreased abundance of Bacteroidetes has been consistently linked to irritable bowel syndrome (Jeffery et al., 2012). The increase in Firmicutes: Bacteroidetes ratio with aging is linked to poorer defense against pathogens and increased inflammatory response (Mariat et al., 2009; Kim et al., 2016).

LPS-treated mice displayed important changes in gut microbial composition 24 hours following LPS treatment compared to saline controls. LPS-treated mice displayed an increase in the gram-negative Proteobacteria and Verrucromicrobia phyla, regardless of age and sex. The Proteobacteria phylum is related to pathogens like *E.coli* and helicobacter, and increased abundance of this phylum is linked to chronic low-grade inflammation (Carvalho et al., 2012). The Verrucromicrobia phylum tends to colonize the gut following antibiotic treatment (Dubourg et al., 2013), suggesting that increases in this phylum could have harmful effects on other phyla

communities within the gut. LPS-treated pubertal mice exhibited a larger decrease in Bacteroidetes, effectively matching saline-treated adults. A loss in the relative abundance of this phylum could suggest increased vulnerability to invading pathogens (Mariat et al., 2009; Kim et al., 2016). These LPS-induced acute changes may result in additional consequences, like changes in gut permeability, which may alter future responses to immune challenges (Maes et al., 2008). Inflammatory markers such as CD14 (an LPS-binding protein), C-reactive protein and NF- κ B become more concentrated in the colon with age (Stehle Jr et al., 2012; Kim et al., 2016), suggesting a low-grade chronic inflammatory state. We also observed sex-specific acute changes in genera with LPS treatment in pubertal mice at 24 hours post-injection, such that, males displayed a greater depletion compared to females. One important bacterium that was significantly depleted by LPS treatment in our adolescent mice was the *Lactobacillus* genera.

The LPS-induced acute changes in gut microbial composition in pubertal mice did not persist into adulthood. Given that the fecal samples were only collected at 24 hours and 8 weeks following LPS treatment, the duration of LPS-induced changes to the gut microbiota is not known. Nonetheless, our results show that these changes are transient. Future studies should investigate age- and sex-specific recovery of the gut microbiota following LPS exposure. Given the transient depletion in *Lactobacillus* and that species of *Lactobacillus* show positive effects on depression- and anxiety-like behavior and stress responses (Messaoudi et al., 2011; Arseneault-Breard et al., 2012; Bravo et al., 2011), we next examined the effect of colonizing the gut with the probiotic *Lactobacillus reuteri* (*L. reuteri*) throughout pubertal development.

We observed sex differences in LPS-induced sickness behaviour in mice exposed to the probiotic *L. reuteri*. Probiotics are known to exert beneficial immunomodulation (Desbonnet et al., 2010). We found that LPS-treated males exposed to *L. reuteri* display less sickness

symptoms at 24 hours compared to broth-fed controls. Despite small mean differences, these findings suggest that *L. reuteri* may facilitate recovery from LPS-induced sickness in males and are consistent with previous findings showing that LPS-induced sickness behaviour and peripheral and central inflammation is decreased in pubertal CD1 male mice following exposure to probiotic strains (Murray et al., 2019a). In contrast, LPS-treated females exposed to *L. reuteri* display more sickness symptoms than broth-fed controls at 24 hours, suggesting that *L. reuteri* had sex-specific effects on sickness behavior. The current results suggest that *L. reuteri* altered the immune response in a sex-specific manner. In addition to *Lactobacillus*, a number of other genera were depleted by LPS treatment in pubertal mice. The effects of supplementing these other bacterial strains on acute and enduring neural and behavioral outcomes require further investigation.

Pubertal exposure to LPS causes enduring anxiety-like behaviour in male mice, as indicated by less time spent in the inner zone of the OFT. However, treatment with *L. reuteri* modulated these effects, as indicated by no difference in the time spent in the inner zone of the OFT between LPS- and saline-treated males. Other findings from our laboratory show subtle changes in locomotion following pubertal probiotic treatment (Yafoufi et al., unpublished observations). Therefore, the saline-treated males exposed to probiotic treatment are the appropriate controls to our LPS-treated males exposed to the probiotic treatment. We observed that *L. reuteri* treatment also reduced anxiety-like behaviour in females that were not exposed to an immune challenge. These findings are in line with previous studies which also observed similar anxiolytic effects of probiotic treatment (Messaoudi et al., 2011; Arseneault-Breard et al., 2012). Gastrointestinal inflammation has been associated with increased anxiety-like behaviour (Bercik et al., 2010). We speculate that LPS-induced transient microbial dysbiosis during

pubertal development which contributed to the enduring negative consequences on anxiety-like behaviours in adulthood. These LPS-induced enduring sex-specific behavioural alterations are likely due to an increased vulnerability to an immune challenge during pubertal development in males. Specifically, pubertal males display greater IL-1 β , TNF α , and IL-6 mRNA expression in the prefrontal cortex at two hours following LPS treatment (Sharma et al., 2018), and show transient increase in BBB permeability at four hours post-LPS administration (Maggioli et al., 2016) compared to females. Treatment with *L. reuteri* mitigated these LPS-induced enduring effects on anxiety-like behaviour in males. There are a number of mechanisms through which *L. reuteri* could have reduced sickness behaviour and mitigated these enduring effects of pubertal LPS treatment in males. For example, Lactobacillus can produce SCFAs, which can inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway, causing less inflammatory reactions (Jeong et al., 2015; Liu et al., 2018). Probiotics can also strengthen tight junctions in the gut, resulting in less permeability and inflammation (Anderson et al., 1993; Arrieta et al., 2006). Moreover, Lactobacillus could have also promoted microglia maturation in the CNS, which is facilitated by microbial-derived SCFAs (Borre et al., 2014; Erny et al., 2015), resulting in less central inflammation to LPS. Nevertheless, females also benefitted from probiotic treatment but in the absence of an immune challenge.

Treatment with *L. reuteri* mitigated enduring effects of LPS treatment on stress-induced brain activation in males. Specifically, pubertal *L. reuteri* treatment mitigated the restraint stress-induced increase in c-Fos expression in the CA1 and CA3 regions of the hippocampus in males treated with LPS during puberty. Interestingly, saline-treated females exposed to *L. reuteri* displayed less c-Fos immunoreactivity in the dentate gyrus (DG) of the hippocampus and less anxiety-like behaviour in the OFT compared to broth fed controls. The DG region of the

hippocampus is the main gateway for signals received via cortical and subcortical projections, including the amygdala (Fa et al., 2014). Reduced c-Fos expression in the DG in these females may suggest that probiotic treatment during puberty altered HPA axis maturation, resulting in less stress-induced excitatory input in the DG in adulthood. Probiotics are known to alter brain function through various pathways, including the HPA axis (Cryan & Dinan, 2012). For example, germ-free (GF; lacking gut microbiota) mice mount an exaggerated stress response (Sudo et al., 2014), but colonizing these mice with commensal microbiota neonatally can partially reverse the sensitization of the HPA axis if exposure occurs early, and not later in neonatal development (Sudo et al., 2004). These findings highlight that microbes may have specific priming effects on brain neurochemistry and HPA axis responsiveness during specific periods of development. A similar effect was found during prenatal development where a single exposure to microbial metabolites (i.e. butyrate and propionate) during late gestation resulted in enduring sex-specific behavioral alterations in adulthood, suggesting that acute changes to the gut microbiota during critical periods of neurodevelopment can permanently alter the functioning of the developing brain (Jašarević et al., 2016). While the microbial changes following probiotic consumption were not examined in the current study, our results suggest that manipulating gut microbial composition with a probiotic can modulate the enduring behavioural and neurochemical alterations induced by pubertal LPS treatment.

Pubertal exposure to LPS did not induce enduring effects on spatial learning and memory. Spatial deficits on the MWM have mostly been found as a result of exposure to chronic variable stress during pubertal development (Avital and Richter-Levin, 2005). The current findings suggest that a single injection of LPS is insufficient to elicit enduring deficits on spatial memory. However, LPS is suggested to affect other cognitive abilities not examined in the

current study, such as the strategies used to learn novel spatial maps on the Barnes Maze (Kolmogorova et al, 2018). We acknowledge that our sample size is small for assessing behavior. However, recent work from our laboratory, using a larger sample size, also failed to find an effect of pubertal LPS treatment on MWM performance (Kolmogorova et al., 2019)

4.1. Conclusions

Our results show that probiotic treatment plays a critical role in modulating the enduring responses to pubertal immune challenge. Given that microbial changes did not persist into adulthood, our findings show that the enduring effects of pubertal LPS treatment on behaviour may be due to acute inflammatory disruptions and changes in gut microbial composition during neurodevelopment, rather than a result of permanent changes in gut microbial diversity and composition. Our research has demonstrated an important influence of gut microbial alterations during pubertal development, a time when stressors are particularly impactful, and draws links to long-term neurobehavioral outcomes. These findings highlight the critical role of the gut microbiome and modulatory effects of specific bacterial strains, such as *L. reuteri* on enduring behavioural and neurochemical outcomes. This research provides evidence into new preventative approaches against stress-related psychological disorders, particularly during sensitive periods of development.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

None.

Supplemental Information

Supplemental Material and Methods

16S-based tag-encoded FLX amplicon pyrosequencing. The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (MR. DNA, Shallowater, TX). Sequences are depleted of barcodes and primers then sequences <200 bp, sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6 bp are all removed. Sequences were then denoised and chimeras removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences, clustering at 3 % divergence (97 % similarity) (Dowd, Callaway et al. 2008; Dowd, Sun et al. 2008; Edgar 2010). OTUs were then taxonomically classified using BLASTn against a curated RDPII/NCBI-derived database (DeSantis, Hugenholtz et al., 2006). Based on the number of sequences per sample, the depth of sequencing was determined to evaluate primary gastrointestinal populations at >0.1 % of the total population.

Morris Water Maze. This task consisted of a circular pool (1.7 m diameter with a rim 50 cm high) filled with water made opaque by non-toxic white tempera paint (Scholar's Choice Inc.). The water depth (30 cm) and temperature ($23\pm 1^{\circ}\text{C}$) were maintained throughout. Landmarks were placed on the walls of the maze. The maze was randomly divided into North, South, East and West quadrants. A hidden platform was placed 1 cm below the surface of the water in the North (target) quadrant.

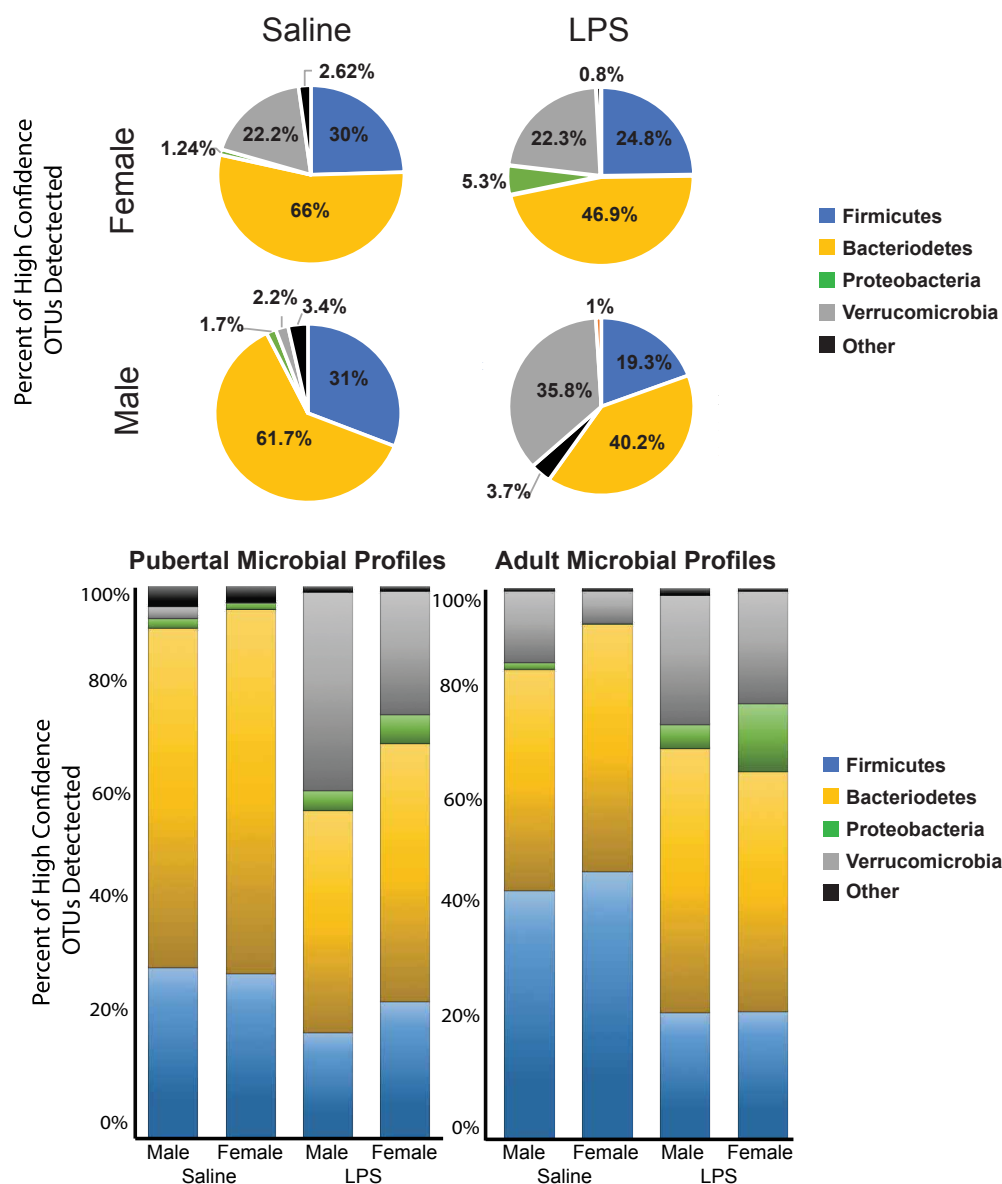


Figure 1. LPS-induced changes in the abundance of bacterial phyla at 24 hours post-LPS treatment in male and female mice. Mean ($\pm$ SEM) relative abundance of each bacterial phylum was analyzed as the average percentage to the total bacterial composition, homogenized from all mice housed in one cage ($n = 10$ mice per treatment group; homogenized samples; $n = 3$). The pie charts illustrate the relative abundance of bacterial phyla in pubertal male and female mice to provide a visual comparison of the saline and LPS-induced treatment differences. The bar graphs summarize the relative abundance of bacterial phyla in pubertal and adult male and female mice treated with saline or LPS. Only the four most abundant phyla are depicted (Firmicutes, Bacteroidetes, Proteobacteria and Verrucomicrobia). All other phyla that are expressed at $<10\%$ in the gut microbiome are represented in the “Other” category (e.g. Candidatus Saccharibacteria, Cyanobacteria, Actinobacteria and Deferribacteres).

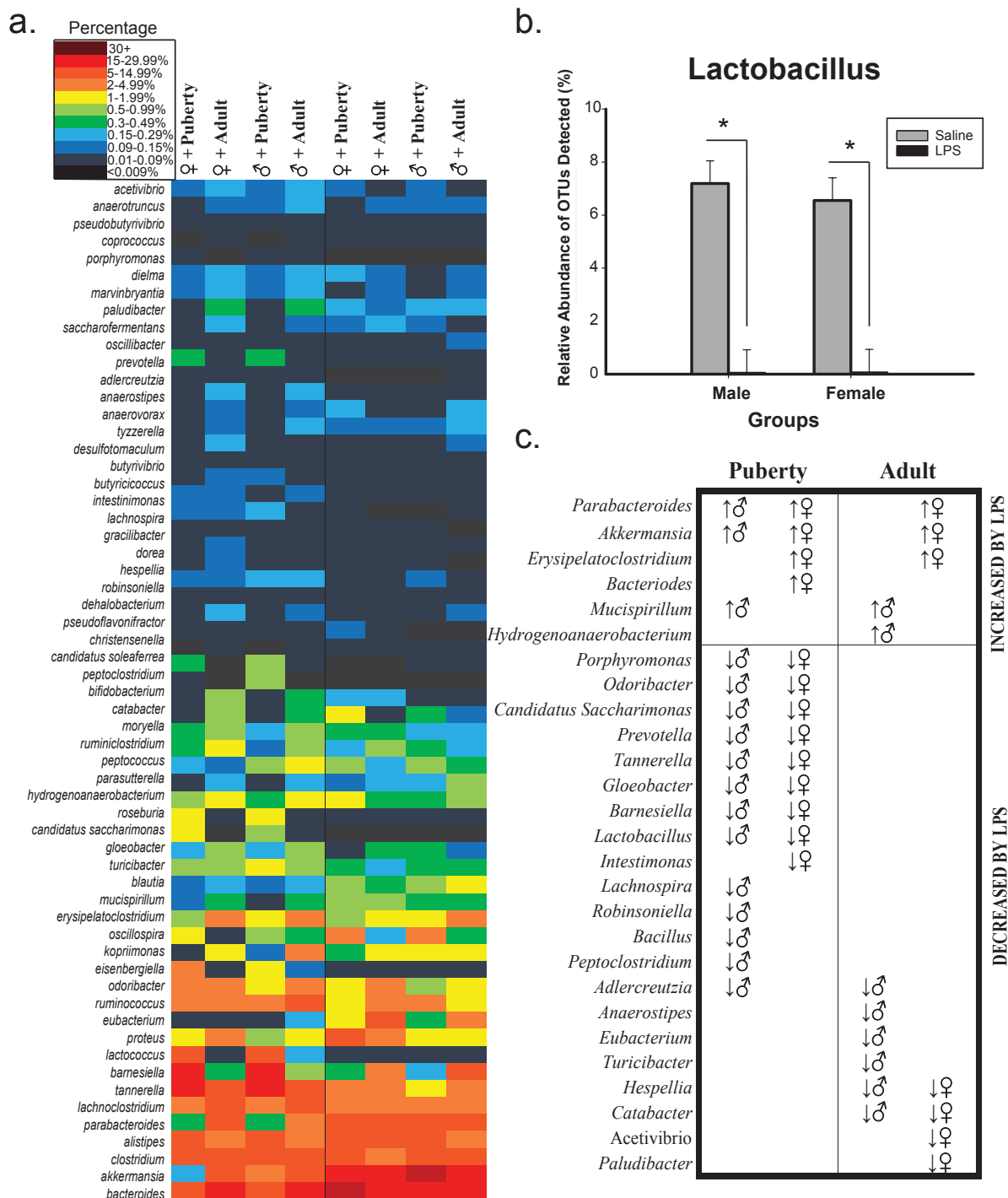


Figure 2. LPS-induced changes in bacterial genera at 24 hours post-LPS treatment in both males and females. (a) Heat Map summarizing gut microbial composition at the genus level for all groups. (b) Relative abundance (%) of *Lactobacillus* in male and female treated with saline or LPS. Data represented as mean ($\pm$ SEM); * $p < 0.05$, $n = 10$ mice per treatment group; homogenized samples; $n = 3$ (c) Diagram depicting a total of 27 genera significantly increased or decreased by LPS treatment, in pubertal and adult male and female mice, $p < 0.05$.

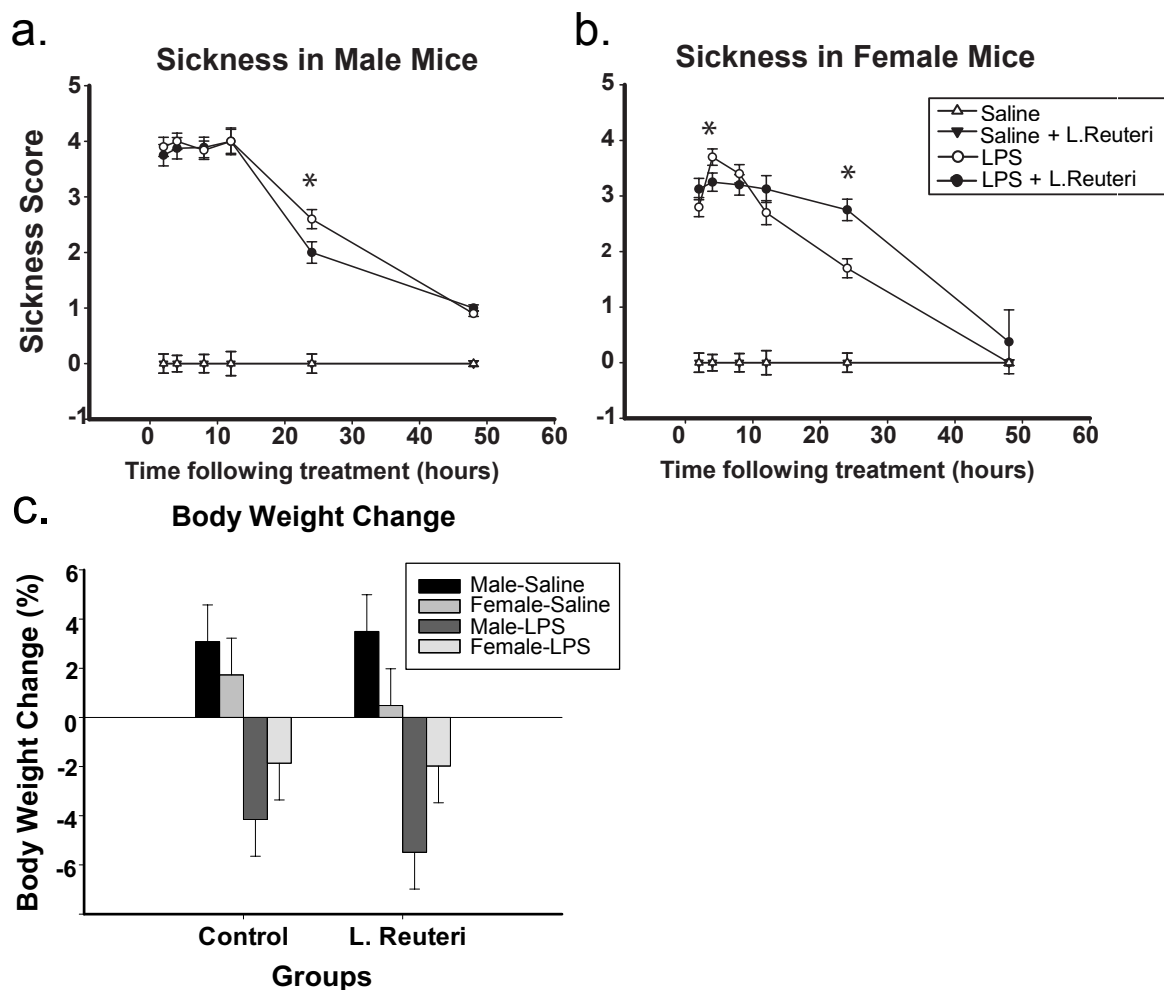


Figure 3. LPS and probiotic (L.Reuteri) treatment during puberty affects sickness behaviour in a sex-specific manner. Sickness scores of six-week-old (a) male and (b) female mice treated with saline or LPS and exposed to either the control broth (Saline, LPS) or L. Reuteri (Saline + L. Reuteri, LPS + L. Reuteri) as a function of time. (c) Acute percent body weight change of male and female mice at 48 hours following saline or LPS treatment and exposed to either control broth or L. Reuteri treatment. Data represented as mean ($\pm$ SEM); * $p < 0.05$, $n = 5$ /group.

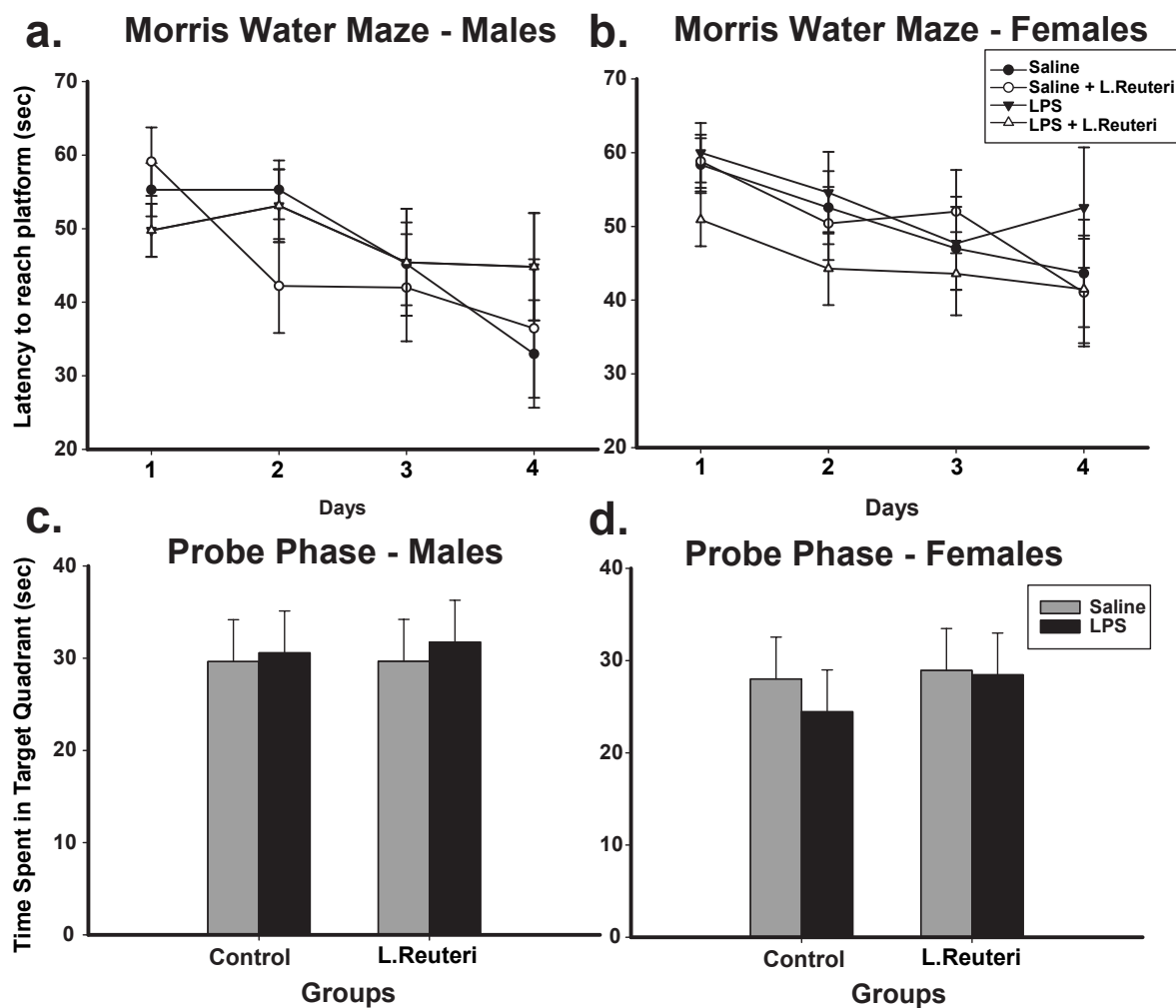


Figure 4. Pubertal LPS treatment does not impact spatial learning. Latency to find the platform (sec) in adult (a) male and (b) female mice previously treated with saline or LPS at 6 weeks of age and exposed to a two-week course of either control broth (Saline, LPS) or *L. Reuteri* (Saline + *L. Reuteri*, LPS + *L. Reuteri*) from 5-7 weeks of age. During the probe phase (day 5), the hidden platform was removed, and the time (sec) the mouse spent in the target (north) and non-target quadrants was recorded in (c) male and (d) female treatment groups. Data represented as mean ($\pm$ SEM); $n = 5/\text{group}$.

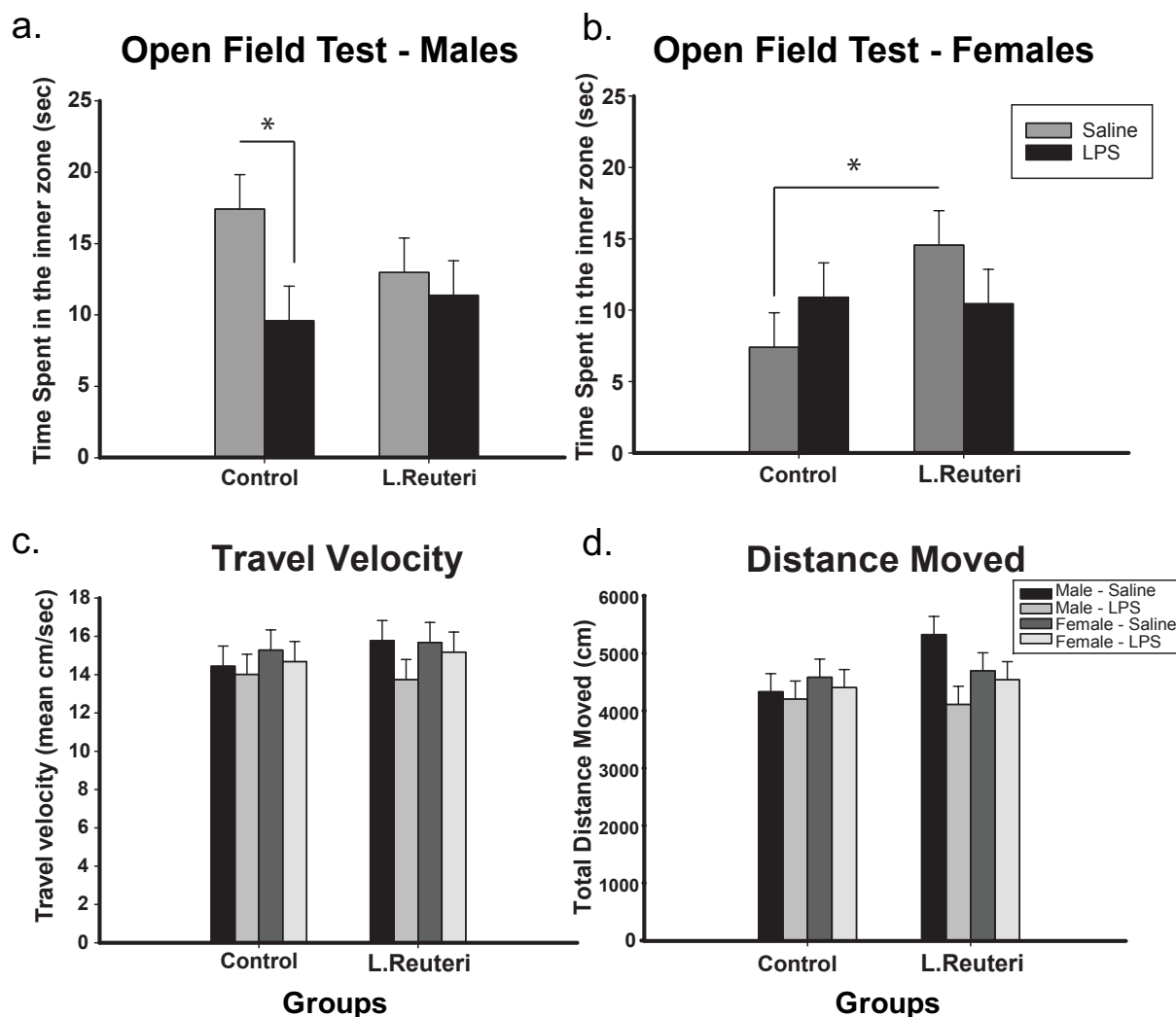


Figure 5. Pubertal exposure to *L. Reuteri* modulates LPS-induced increases in anxiety-like behavior in males and tends to decrease stress-reactivity in females. Time spent (sec) in the inner zone of the arena of the Open Field Test (OFT) in adult (a) male and (b) female mice previously treated with saline or LPS at 6 weeks of age and exposed to a two-week course of either control broth or *L. Reuteri* treatment (from 5-7 weeks of age). Measures of locomotion; (c) travel velocity (mean cm/second) and (d) distance travelled (cm) are represented for all treatment groups. Data represented as mean ($\pm$ SEM); * $p < 0.05$; # $p < 0.058$, $n = 5$ /group.

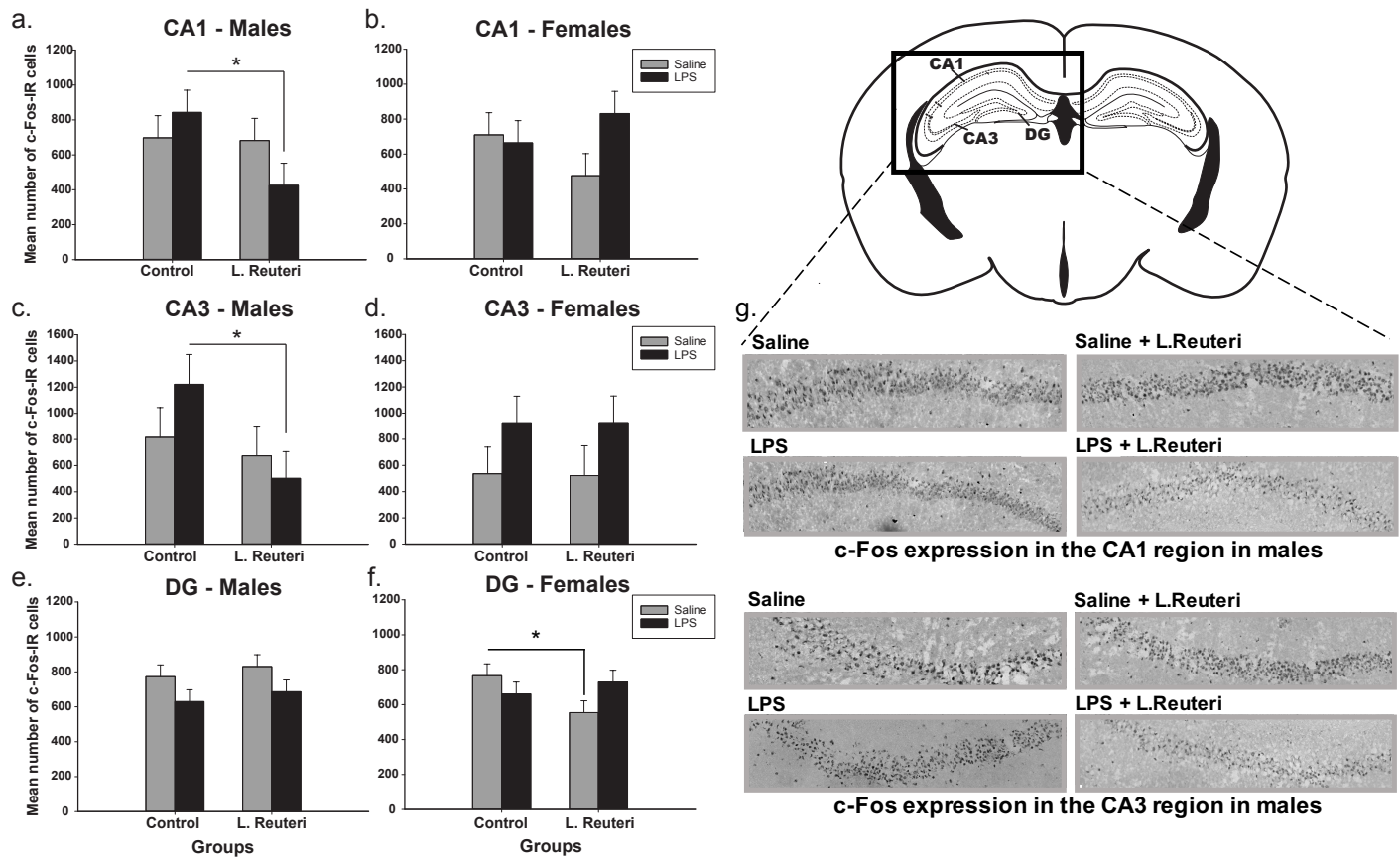
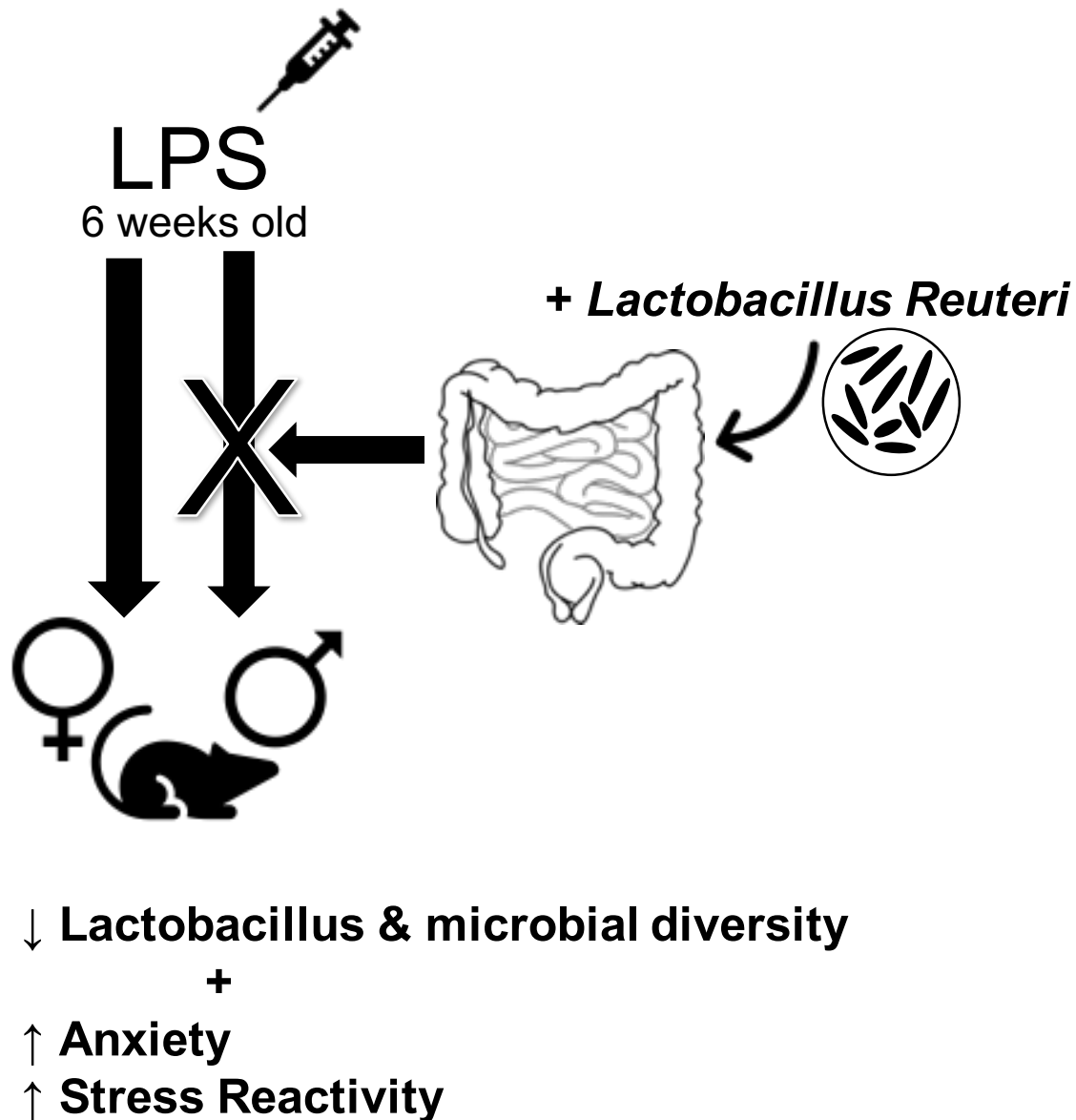


Figure 6. Pubertal exposure to *L. Reuteri* treatment mitigates stress-induced increases in c-Fos expression in the CA1 and CA3 regions of the hippocampus in males in adulthood. Expression of c-Fos immunoreactive cells one hour following restraint stress in adult (a) male and (b) female mice previously treated with saline or LPS at 6 weeks of age and exposed to a two-week course of either control broth or *L. Reuteri* treatment. Data represented as mean ($\pm$ SEM); * $p < 0.05$, $n = 5$ /group.



Supplemental Figure. Model depicting treatment with *L. Reuteri* during puberty modulates stress-induced anxiety-like behavior and increases in stress reactivity. At six weeks of age, immune challenge (LPS) prompted changes in microbial composition and enduring effects on anxiety-like behaviour into adulthood. Replenishment of the gut with *L. Reuteri*, modulates the detrimental effects on anxiety and stress reactivity thereby promoting resilience against inflammatory-induced mental health outcomes.

Article 3

Age-specific gut microbiota regulate LPS-induced immune responses.

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Abstract

Puberty is a critical period of development that is accompanied by the maturation of the stress and immune systems. There are marked age and sex differences in peripheral and central inflammatory responses to an immune challenge between pubertal and adult mice. Given the strong link between the gut microbiome and immune system, it is possible that the age and sex differences in immune responses are mediated by age and sex differences in gut microbial composition. The current study investigated whether the transfer of gut microbiota between adult and pubertal CD1 mice, through three weeks of pair-housing, could mitigate age- and sex-specific immune responses. Cytokine concentrations in the blood and cytokine mRNA expression in the brain were assessed following exposure to the immune challenge lipopolysaccharide (LPS). The results show that all mice displayed increased cytokine concentrations in serum and central cytokine mRNA expression in the hippocampus, hypothalamus and prefrontal cortex (PFC) at eight hours following LPS treatment. Pubertal male and female mice, that were pair-housed with a pubertal counterpart, displayed lower cytokine concentrations in serum and lower cytokine mRNA expression in the brain compared to adult mice that were pair-housed with an adult counterpart. However, when adult and pubertal mice were pair-housed and underwent microbial exchange, the age and sex differences in both peripheral cytokine concentrations and central cytokine mRNA expression were mitigated. These results show that microbial composition modulates immune response and that the age difference in immune response can be mitigated by microbial exchange between pubertal and adult mice.

Keywords: Puberty, Stress, Gut microbiota, Sex differences, Age differences, Mice, LPS, Immune responses

1. Introduction

The immune system is a complex and multifaceted system responsible for the recognition and the response to pathogens (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2000). There are marked age and sex differences in both peripheral and central inflammatory responses to an immune challenge between pubertal and adult mice (Cai et al., 2016; Girard-Joyal et al., 2015; Sharma et al., 2018). For example, pubertal CD1 mice exposed to lipopolysaccharide (LPS), a bacterial endotoxin, display less hypothermia (Cai et al., 2016) and reduced c-Fos expression in a number of brain regions compared to adults two hours following LPS treatment (Girard-Joyal et al., 2015). Moreover, pubertal male mice show greater LPS-induced central IL-6, IL-1 β and TNF α mRNA expression in the prefrontal cortex (PFC) compared to both pubertal female and adult male counterparts two hours following LPS treatment. At eight hours following LPS treatment, pubertal females display significantly more IL-6, IL-1 β and TNF α mRNA expression in the hippocampus compared to their pubertal male and adult female counterparts (Sharma et al., 2018). Pubertal mice also display more anti-inflammatory cytokines and less pro-inflammatory cytokines compared to their adult counterparts, at ten hours post-infection (Cai et al., 2016). The mechanisms underlying these age- and sex-specific immune responses remain elusive. One system that could provide insight into these age and sex differences is the gut microbiome.

The gut microbiome is a diverse and dynamic system comprised of trillions of microorganisms and contributes to the regulation of immune responses both peripherally and within the central nervous system (CNS; Belkaid & Hand, 2014; Erny et al., 2015; Fung, Olson & Hsiao, 2017; Mu et al., 2016). The gut microbiome communicates with the CNS through immune (e.g. cytokines), neural (e.g. vagus and enteric nervous system), and endocrine (e.g.

cortisol) pathways to impact brain biochemistry and immune responses (Cryan & Dinan, 2012; Fung et al., 2017). Commensals (i.e. microbiota in a symbiotic relationship with the host) and pathogenic microbes can activate the host immune system through the production of microbial metabolites (e.g. short-chain fatty acids; SCFAs) and co-metabolites (e.g. liposaccharide). These metabolites interact with epithelial cells on the mucosal cell surface (Rooks & Garrett, 2017) and activate dendritic cells (DCs) that reside in the intestine. Activation of DCs in response to microbial metabolites, leads to the production of chemokines, antimicrobial peptides and cytokines (Kinnebrew et al., 2012; Uematsu et al., 2008; Uematsu et al., 2006). Moreover, gut microbes are essential for the development of immunocompetent cells in the brain. For example, mice raised under germ free (GF) conditions display significant anomalies in microglia morphology (i.e. an essential immune cell in the CNS) (Erny et al., 2015). Taken together, intestinal microbes are essential for the modulation of peripheral immune responses and maturation of immune cells within the CNS. Given that gut microbiota influence immunity and inflammation (Belkaid & Hand, 2014; Erny et al., 2015; Fung, Olson & Hsiao, 2017; Mu et al., 2016), it is possible that changes to gut microbial composition could alter age and sex differences in the immune response to an invading pathogen.

The diversity and composition of the gut microbiome changes across the lifespan. During early postnatal development, the gut microbiome contains a limited number of genera, which increase steadily during the first year of life (Bakhed et al., 2015). The gut microbiome continues to develop and mature throughout childhood and adolescence (Palmer et al., 2007; Yatsunen et al., 2012). These major transitions in microbial composition tend of parallel dynamic critical periods in brain development (Borre et al., 2014; Jasarevic et al., 2015). The structure and diversity of the microbiome is largely stable by early adulthood but fluctuates with stress,

infection, diet and antibiotics (Spor, Koren & Ley, 2011). A large cross-sectional study revealed that adult males exhibit greater abundance of *Bacteroides* and *Prevotella* compared to adult females, suggesting that gonadal steroid hormones may influence gut microbial composition (Mueller et al., 2006). Recent work from our laboratory shows that pubertal CD1 mice display greater diversity in the gut microbiota compared to adult counterparts, suggesting that gut microbial diversity likely peaks during puberty and then declines with aging. Compared to adult mice, pubertal mice display greater changes in microbial composition following exposure to LPS (Murray et al., 2019a), suggesting greater gut microbial vulnerability to pathogens during this critical period in neurodevelopment. However, the extent to which age-specific gut microbial taxa can regulate inflammatory responses remains uninvestigated.

The current study examined the effects of microbial exchange between pubertal and adult mice, through natural coprophagic activity, on peripheral and central inflammatory responses to an immune challenge. Coprophagy refers to the consumption of fecal excrement, a natural behaviour among mice and other animals. In mice, coprophagy has important nutritional benefits as it provides a source of vitamins, minerals, amino acids, and other nutrients, which are not effectively absorbed by the gastrointestinal (GI) tract (Soave & Brand, 1991). We hypothesized that colonizing the gut of pubertal mice with microbes from adults would cause the pubertal mice to display an LPS-induced immune response that is similar to that of adult counterparts, as evidenced by greater concentrations of pro-inflammatory cytokine in blood serum and greater central cytokine mRNA expression. We also hypothesized that colonizing the gut of adult mice with microbes from pubertal mice would cause adult mice to exhibit lower concentrations of pro-inflammatory cytokines and less cytokine mRNA expression. Lastly, we hypothesized that

microbial transfer from adult to pubertal mice would affect pubertal development and maturation and result in earlier vaginal opening in females and faster scrotal growth in males.

2. Methods

2.1. Animal Housing

80 male and 80 female CD-1 mice were shipped from Charles River Laboratories (St-Constant, Québec) at three weeks of age. Mice were pair-housed in Polycarbonate Lexan cages (17 X 28 X 12 cm [width X length X height]) in either an all-male or all-female colony room with *ad libitum* access to food (Harlan Laboratories, Inc., Madison, WI, US, T2018 – Global 18% rodent) and water. Housing rooms were kept at constant temperature ($24 \pm 2^{\circ}\text{C}$; relative humidity of 40 ± 5) with a reversed light:dark cycle (14:10; lights off at 10:00am). All procedures were approved by the Animal Care Committee of the University of Ottawa.

2.1.1. Pair-housing and Experimental Procedures

Pair-housing with a same-sex partner began at either 3-weeks-of-age (pubertal group) or 7-weeks-of-age (adult group). Pubertal mice were either pair-housed with an age-matched cage mate or an adult mouse. Similarly, adult mice were either pair-housed with an age-matched cage mate or a pubertal mouse. The pair-housing conditions generated four groups: adult mice pair-housed together (AA), adult mice pair-housed with pubertal mice (AP), pubertal mice pair-housed with adult mice (PA), and pubertal mice pair-housed together (PP). Mice were pair-housed for a total of three weeks to promote coprophagic activity.

2.1.2. Vaginal Opening Examination.

Vaginal opening is used as an external index of pubertal onset. The evaluation was conducted by two raters blinded to treatment conditions. Visual observation of vaginal opening was conducted daily by gently lifting the female by the tail and confirming opening of the vulva (Caligioni,

2010). Once vaginal opening was identified, the mouse was checked again the following day to confirm the decision.

2.1.3. Scrotum Width Measurements

An analog caliper was used to measure the scrotum width. The caliper was placed around the scrotum so as to only slightly pinch it. The evaluation was conducted for two weeks from the onset of pair-housing. Scrotum width was recorded every second day.

2.2. Immune Challenge

2.2.1. Lipopolysaccharide (LPS)

Lipopolysaccharide (obtained from *Escherichia coli* serotype O26:B6; #L3755; Sigma Aldrich Canada, Oakville, ON) was prepared by dissolving lyophilized LPS into 0.9% (w/v) sterile aqueous sodium chloride (saline) solution (Ricca Chemical Company; #R7210000-4A7210-1). All mice were injected intraperitoneally at six (pubertal groups) or ten (adult groups) weeks of age at the end of the light cycle with either 0.9% sterile saline or LPS (1.5mg/kg). This dose of LPS causes mild sickness in mice for up to 48 hours (Cai et al., 2016; Ismail, Garas & Blaustein, 2011). When administered at six weeks of age, LPS produces long-term impairments in reproductive and non-reproductive behaviors (Laroche et al., 2009; Ismail, Kumlin, Blaustein, 2013; Komogorova et al., 2019). In order to minimize additional manipulation and handling stress to females, vaginal smears were not collected. Given that estrous cycling is known to begin around nine weeks of age in CD1 mice housed in similar conditions, we did not expect that our six weeks old mice were cycling yet. All mice received an intraperitoneal injection of Euthanyl (500 mg/kg body weight; prepared from Euthansol; Merck Animal Intervet Canada Corp; Kirckland, Quebec) at eight hours following saline or LPS injection to examine the effects

of sex and pair-housing conditions on LPS-induced inflammatory responses in blood serum and brain.

2.2.2. Multiplex Luminex Immunoassay

Trunk blood was collected into Microvette CB 300 Z capillary tubes and stored at 4°C overnight. Tubes were centrifuged at 10,000g at 20°C, the following day, for five minutes to separate blood serum. Serum was then stored at -80°C until analysis. A multiplex Luminex immunoassay was conducted to measure concentrations of the interleukins (IL)-1 β , 6, 10, 12(p70), interferon- γ (IFN γ), and tumour necrosis factor α (TNF α). Serum was diluted in assay buffer solution, by a factor of two. Serial dilutions were prepared for mouse cytokine standards at concentrations of 3.2, 12, 80, 400, 2000 and 10,000 pg/ml. Then, a cytokine bead solution was added to the plates in all wells containing the standards, samples, and controls. Next, the plates were sealed and placed on a shaker at 4°C. Plates were left to incubate for 16 hours. After washing, detection antibodies were added to each well, and the plates were sealed and incubated on a plate shaker at room temperature for one hour. Streptavidin-phycoerythrin was added to all wells and then incubated on a plate shaker at room temperature for 30 min. Plates were washed and beads were re-suspended in drive fluid. Reading was conducted on a MAGPIX system to measure cytokine concentrations. Intra-assay coefficient of variation (CV%) of the duplicate wells ranged from 0.5-19.9% and inter-assay was <15% for each cytokine concentration. Due to large intra-assay coefficient between duplicate wells, these two samples were removed prior to statistical analysis.

2.2.3. Real-time qPCR

Following euthanasia, brains were extracted and immediately frozen using liquid nitrogen, and stored at -80°C. Brains were dissected to collect the hippocampus, and hypothalamus and prefrontal cortex (PFC), following guidelines from *The Mouse Brain Atlas in Stereotaxic*

Coordinates (Franklin and Paxinos, 1997). Isol-RNA lysis Reagent (Cat. No. 2302700, Fisher Scientific) was used to extract messenger RNA (mRNA) from frozen brain tissue. Extracted RNA was treated with DNase in order to remove genomic DNA prior to cDNA synthesis using the QuantiTect Reverse Transcription kit (Cat. No. 205311, Qiagen). Resulting cDNA aliquots were used to identify the relative gene expression in the samples. Gene expression was assessed using the RealMasterMix Fast SYBR kit (Cat. No. 1725201, Bio-Rad) in 10 μ L reactions on the CFX96TOUCH real time PCR machine. Efficiencies of the primers (ordered from Integrated DNA Technologies) was determined using the slope of the relation between RNA quantity and cycle thresholds (C_T) using Bio-Rad software technology. Standard curves revealed that all primer pairs achieved reaction efficiencies between 90% and 110%. Primers were diluted to a final concentration of 0.3 μ M for the real-time PCR reaction. The sequences of primers were as follows (Table 1):

Table 1. Summary of Primer sequences.

Target Gene	Forward	Reverse
β -actin	GAACCCTAAGGCCAACCGTG	GGTACGACCAGAGGCATACAGG
IL-1 β	TCTTGGGACTGATGCTGGTG	CAGAATTGCCATTGCACA ACTC
TNF α	GCCTATGTCTCAGCCTCTTCTC	GCCATTTGGGAAGCTTCTCATCC
IL-6	GCCTTCTTGGGACTGATGCT	GCCATTGCACAACTCTTTTCTC

β -actin was used as the reference/housekeeping gene for all samples. No significant difference in β -actin mRNA expression was found between experimental groups. For each reaction, the quantitative threshold amplification cycle number (C_q) was determined by taking the average across groups, and the $2^{-\Delta\Delta C_q}$ equation was used to calculate the relative gene expression.

2.3. Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics (version 22). Outliers were identified using Z-scores and data that was 1.96 SD away from the mean was considered an outlier (Tabachnick & Fidell, 2007). Windsorizing was performed to limit extreme values from the analyses (Dixon, 1960). A maximum of two serum cytokine concentrations were windosorized within each treatment group. In regard to mRNA cytokine expression, a maximum of two cytokines were windosorized within each treatment group. A t-test was conducted to examine differences in vaginal opening between pubertal females pair-housed with either a pubertal or an adult female. A two-way mixed ANOVA was used to examine the effect of pair-housing on scrotum width in males across time. Three-way between-subject ANOVAs were used to examine the effects of sex, LPS treatment and pair-housing condition on peripheral cytokine concentration and central cytokine mRNA expression. Significant interactions were followed by pairwise comparisons and a Bonferroni correction factor was applied to control for the number of comparisons. The criterion for statistical significance was set at $p < 0.05$.

3. Results

3.1. Vaginal Opening.

There was a significant effect of pair-housing on vaginal opening in pubertal mice ($t_{(13)} = 17.27$, $p < 0.001$). Pubertal females that were pair-housed with an adult female (PA) showed earlier vaginal opening ($M = 10.75$, $SD = 1.39$) compared to pubertal females pair-housed with another pubertal female (PP) ($M = 7.00$, $SD = 2.00$) (Fig 1a).

3.2. Scrotum Width.

A three-way ANOVA revealed a main effect of time ($F_{(4,15)} = 26.57$, $p < 0.001$, $\eta^2_p = 0.356$), and a significant time x pair-housing interaction ($F_{(1,15)} = 13.03$, $p = 0.001$, $\eta^2_p = 0.449$) on scrotum

width. All adult males showed significantly larger scrotum sizes compared to pubertal males across all time-points ($p < 0.001$). Pairwise comparisons revealed that pubertal males housed with an adult male (PA) showed larger scrotum size than pubertal mice housed with a pubertal counterpart (PP) at four (MD = 0.675, SE = 0.147, $p < 0.001$), six (MD = 0.592, SE = 0.148, $p < 0.001$), eight (MD = 0.617, SE = 0.152, $p = 0.001$), ten (MD = 0.442, SE = 0.151, $p < 0.05$) and 12 (MD = 0.467, SE = 0.141, $p < 0.05$) days of pair-housing (Fig 2a).

3.3. Body Weight.

A three-way ANOVA revealed a main effect of time in males ($F_{(6,535)} = 387.92$, $p < 0.001$, $\eta^2_p = 0.890$), and females ($F_{(3,70)} = 118.02$, $p < 0.001$, $\eta^2_p = 0.847$) and a main effect of age for females ($F_{(1,26)} = 31.640$, $p < 0.001$, $\eta^2_p = 0.549$) and males ($F_{(1,26)} = 198.886$, $p < 0.001$, $\eta^2_p = 0.833$) on body weight. There was also a significant time X cohabitation condition interaction in males ($F_{(6,535)} = 56.99$, $p < 0.001$, $\eta^2_p = 0.781$) and females ($F_{(3,70)} = 16.89$, $p < 0.001$, $\eta^2_p = 0.679$) on body weight. As expected, all adult male and female mice weighed significantly more than their pubertal counterparts mice across all time points ($p < 0.001$). Pubertal males that were pair-housed with an adult weighed less than pubertal males pair-housed with a pubertal on day four of pair-housing (MD = 4.743, SE = .931, $p < .001$) (Fig 2b).

3.4. Age and sex differences in peripheral pro- and anti-inflammatory cytokine concentrations following pair-housing and LPS treatment

Three-way ANOVA revealed main effects of LPS treatment on IL-6 ($F_{(1,132)} = 97.072$, $p < 0.001$, $\eta^2_p = 0.424$), TNF α ($F_{(1,131)} = 229.505$, $p < 0.001$, $\eta^2_p = 0.637$), IL-1 β ($F_{(1,132)} = 175.616$, $p < 0.001$, $\eta^2_p = 0.571$), IFN γ ($F_{(1,132)} = 225.335$, $p < 0.001$, $\eta^2_p = 0.631$), IL-12 ($F_{(1,131)} = 198.056$, $p < 0.001$, $\eta^2_p = 0.602$), and IL-10 ($F_{(1,132)} = 88.971$, $p < 0.001$, $\eta^2_p = 0.403$) concentrations in the serum. There was a main effect of pair-housing on IL-6 ($F_{(3,132)} = 3.338$, $p = 0.021$, $\eta^2_p = 0.071$),

IL-1 β ($F_{(3,132)} = 4.484, p = 0.005, \eta^2_p = 0.092$), IFN γ ($F_{(3,132)} = 6.477, p < 0.001, \eta^2_p = 0.128$), and IL-12 ($F_{(3,131)} = 5.219, p = 0.002, \eta^2_p = 0.107$) concentrations. There was a main effect of sex on IL-10 concentration ($F_{(1,131)} = 6.209, p = 0.014, \eta^2_p = 0.045$). Statistical analyses also revealed a significant pair-housing X LPS treatment interaction on IL-6 ($F_{(3,132)} = 3.334, p = 0.022, \eta^2_p = 0.070$), IL-1 β ($F_{(3,132)} = 4.551, p = 0.005, \eta^2_p = 0.094$), IFN γ ($F_{(3,132)} = 6.477, p < 0.001, \eta^2_p = 0.128$), IL-12 ($F_{(3,131)} = 5.393, p = 0.002, \eta^2_p = 0.110$) concentrations in the serum. There was also a significant sex X LPS treatment interaction on IL-12 ($F_{(3,131)} = 8.004, p = 0.005, \eta^2_p = 0.058$) and IL-10 ($F_{(3,131)} = 6.272, p = 0.013, \eta^2_p = 0.045$) concentrations.

As expected, pairwise comparisons revealed that LPS-treated mice showed greater concentrations of IL-6, TNF α , IL-1 β , IFN γ , IL-12 and IL-10 compared to saline-treated controls across all treatment conditions ($p < 0.05$). LPS-treated adult males pair-housed with another adult male (AA) showed greater IL-6 concentration compared to LPS-treated adult females pair-housed with an age-matched female counterpart (AA) (MD = 8217.674, SE = 3621.517, $p = 0.025$). However, there was no sex difference in IL-6 concentration between LPS-treated adult male and female mice that were pair-housed with a pubertal mouse (AP). Compared to LPS-treated AA males, LPS-treated pubertal males pair-housed with another pubertal male (PP) showed lower concentrations of TNF α (MD = 38.839, SE = 12.631, $p = 0.015$), IL-6 (MD = 14370.667, SE = 3637.018, $p = 0.001$), IL-12 (MD = 48.150, SE = 7.978, $p < 0.001$) and IL-1 β (MD = 51.386, SE = 13.424, $p = 0.002$). However, the age difference in TNF α , IL-12 and IL-1 β and IL-6 concentrations were absent in pubertal males' pair-housed with an adult male (PA) (Fig 3b, c, e). Compared to LPS-treated PP females, LPS-treated AA females showed greater concentrations of IFN γ (MD = 3900.269, SE = 1238.620, $p = 0.012$) and IL-10 (MD = 521.956, SE = 108.585, $p < 0.001$). However, LPS-treated AP female mice did not significantly differ

from LPS-treated PA females in $\text{IFN}\gamma$ (MD = 1173.498, SE = 1229.716, $p = 1.00$) and IL-10 (MD = 34.293, SE = 103.121, $p = 1.00$) concentrations (Fig 3g, j).

3.5. Pro- and anti-inflammatory cytokines mRNA expression in the hypothalamus, hippocampus and prefrontal cortex following LPS treatment

3.5.1 Interleukin-1 Beta (IL-1 β) mRNA expression.

Three-way ANOVA revealed main effects of LPS treatment on IL-1 β mRNA expression in the PFC ($F_{(1,72)} = 36.667$, $p < 0.001$, $\eta_p^2 = 0.337$), hippocampus ($F_{(1,76)} = 46.935$, $p < 0.001$, $\eta_p^2 = 0.382$), and hypothalamus ($F_{(1,75)} = 102.294$, $p < 0.001$, $\eta_p^2 = 0.577$). A main effect of pair-housing was found on IL-1 β mRNA expression in the PFC ($F_{(1,72)} = 3.257$, $p = 0.026$, $\eta_p^2 = 0.120$) and hippocampus ($F_{(1,76)} = 3.825$, $p = 0.013$, $\eta_p^2 = 0.131$).

Pairwise comparisons revealed that LPS-treated PP females showed greater IL-1 β mRNA expression in the PFC compared to saline-treated controls (MD = 2.841, SE = 0.995, $p = 0.006$). LPS-treated AA males showed greater IL-1 β mRNA expression compared to LPS-treated PP males in the PFC (MD = 3.714, SE = 0.995, $p = 0.002$) (Fig 4a), hippocampus (MD = 5.108, SE = 1.914, $p = 0.05$) (Fig 5a) and hypothalamus (MD = 4.137, SE = 1.217, $p = 0.006$) (Fig 6a). LPS-treated AA males also showed greater IL-1 β mRNA expression in the PFC compared to LPS-treated AA females (MD = 2.744, SE = 0.995, $p = 0.007$). Age and sex differences were not present in LPS-treated AP mice. In the hypothalamus, all LPS-treated mice showed greater IL-1 β mRNA expression compared to saline-treated controls ($p < 0.05$). Pubertal LPS-treated PP females showed greater LPS-induced increases in IL-1 β mRNA expression compared to LPS-treated PP males (MD = 2.673, SE = 1.160, $p = 0.024$). In the hippocampus, LPS-treated AP males showed less IL-1 β mRNA expression compared to LPS-treated AA males (MD = 6.354, SE = 1.914, $p = 0.008$) (Fig 6a). LPS-treated AA females showed greater IL-1 β mRNA

expression in the hippocampus compared to LPS-treated AP females (MD = 5.408, SE = 1.914, $p = 0.036$) (Fig 6b).

3.5.2. *Tumor necrosis factor alpha (TNF α) mRNA expression.*

Three-way ANOVA revealed main effects of LPS treatment on TNF α mRNA expression in the PFC ($F_{(1,72)} = 75.674$, $p < 0.001$, $\eta_p^2 = 0.512$), the hypothalamus ($F_{(1,74)} = 168.089$, $p < 0.001$, $\eta_p^2 = 0.694$) and the hippocampus ($F_{(1,61)} = 11.363$, $p = 0.001$, $\eta_p^2 = 0.157$). A sex X injection X pair-housing interaction ($F_{(1,72)} = 3.519$, $p = 0.019$, $\eta_p^2 = 0.128$) was found on TNF α mRNA expression in the PFC.

Pairwise comparisons revealed that LPS-treated AA males showed greater TNF α mRNA expression compared to LPS-treated AA females (MD = 3.962, SE = .990, $p < 0.001$) in the PFC. In contrast, LPS-treated PP females showed greater TNF α mRNA expression in the PFC compared to LPS-treated PP males (MD = 2.275, SE = .944, $p = 0.019$). LPS-treated AA males showed greater TNF α mRNA expression compared to LPS-treated PP males in the PFC (MD = 5.913, SE = .990, $p < 0.001$) (Fig 4c) and the hypothalamus (MD = 2.590, SE = .673, $p = 0.001$) (Fig 5c). However, LPS-treated AP males did not differ from PA males in TNF α mRNA expression in the PFC (MD = .961, SE = 1.056, $p = 1.00$) (Fig 4c), hypothalamus (MD = .362, SE = .745, $p = 1.00$) (Fig 5c), hippocampus (MD = .044, SE = 1.105, $p = 1.00$) (Fig 6c).

3.5.3. *Interleukin-6 (IL-6) mRNA expression.*

Three-way ANOVA revealed main effects of LPS treatment on IL-6 mRNA expression in the PFC ($F_{(1,73)} = 79.683$, $p < 0.001$, $\eta_p^2 = 0.522$), hypothalamus ($F_{(1,76)} = 121.957$, $p < 0.001$, $\eta_p^2 = 0.616$), and hippocampus ($F_{(1,72)} = 26.945$, $p < 0.001$, $\eta_p^2 = 0.272$). A main effect of pair-housing condition ($F_{(1,73)} = 5.517$, $p = 0.002$, $\eta_p^2 = 0.185$) and a significant LPS treatment x pair-housing interaction ($F_{(3,73)} = 5.636$, $p = 0.002$, $\eta_p^2 = 0.188$) were also found on IL-6 mRNA

expression in the PFC. A sex x LPS treatment x pair-housing interaction ($F_{(3,76)} = 3.582, p = 0.018, \eta_p^2 = 0.124$) was found for IL-6 mRNA expression in the hypothalamus.

Pairwise comparisons revealed that in the PFC, LPS-treated AA males showed more IL-6 mRNA expression compared to LPS-treated AA females (MD = 5.304, SE = 1.732, $p = 0.003$). In contrast, LPS-treated PP females show more IL-6 mRNA expression in the PFC compared to LPS-treated PP males (MD = 4.229, SE = 1.652, $p = 0.013$). However, sex differences in IL-6 mRNA expression following LPS treatment were eliminated in PA and AP female mice. LPS-injected AA males showed more IL-6 mRNA expression compared to LPS-treated PP males in the PFC (MD = 9.247, SE = 1.732, $p < 0.001$). LPS-treated AP males showed less IL-6 mRNA expression compared to LPS-treated AA males (MD = 7.846, SE = 1.732, $p < 0.001$). LPS-treated AA males displayed greater IL-6 mRNA expression in the PFC compared to LPS-treated PP males (MD = 5.461, SE = 1.127, $p < 0.001$) (Fig 4e). However, LPS-treated PA males did not differ from their adult counterparts. In the hypothalamus, LPS-treated AA males showed greater IL-6 mRNA expression compared to LPS-treated PP males (MD = 5.461, SE = 1.127, $p < 0.001$) and LPS-treated AA females (MD = 3.947, SE = 1.127, $p = 0.001$). Moreover, LPS-treated AP males showed less IL-6 mRNA expression in the hypothalamus compared to LPS-treated AA males (MD = 5.692, SE = 1.127, $p < 0.001$) (Fig 5e). In the hippocampus, LPS-treated AA females showed greater IL-6 mRNA expression compared to their saline-treated counterparts (MD = 12.576, SE = 2.914, $p < 0.001$; MD = 6.471, SE = 2.914, $p < 0.03$). Moreover, LPS-treated AA females showed greater IL-6 mRNA expression in the hippocampus compared to LPS-treated PP females (MD = 8.854, SE = 2.914, $p = 0.02$). This age difference was not present between the PA and AP females that were pair-housed (MD = .294, SE = 2.817, $p = 1.00$) (Fig 6f).

4. Discussion

There are marked age and sex differences in immune response between pubertal and adult male and female CD1 mice (Cai et al., 2016; Girard-Joyal et al., 2015). However, the mechanisms underlying these age and sex differences are poorly understood. The gut microbiome is an important system that shapes host physiology, including the development and maturation of the immune system (Fung et al., 2017; Jašarević et al., 2015). Recently, our laboratory has demonstrated significant age and sex differences in gut microbial composition between pubertal and adult CD1 mice, and in the microbial changes following an immune challenge (Murray et al., 2019b). Given that gut microbes exert a significant influence on the immune system, we hypothesized that microbial transfer between pubertal and adult mice would change the immune response and mitigate age differences in immune response. To test this hypothesis, pubertal and adult mice were pair-housed for three weeks to facilitate microbial exchange through coprophagic activity prior to the immune challenge.

The results of the current study replicate our previously published work (Cai et al., 2016; Sharma et al., 2018) demonstrating that LPS treatment elicits different immune responses peripherally (i.e. circulating cytokine concentrations) and centrally (cytokine mRNA expression in the brain), in an age- and sex-specific manner. Following exposure to LPS, adult males display significantly greater IFN γ , IL-1 β , TNF α , IL-12 and IL-6 concentrations compared to pubertal males. Moreover, adult females display greater IFN γ concentration compared to pubertal females, while pubertal females display greater concentration of the anti-inflammatory cytokine IL-10 compared to adult females. LPS also triggers inflammation within the CNS (Bilbo & Schwarz, 2009; Kentner et al., 2010). We observe significant age differences in IL-1 β , TNF α and IL-6 mRNA expression, in all three brain regions examined. More specifically, LPS-treated adult

males display greater expression of IL-1 β , TNF α and IL-6 mRNA expression in the PFC and hypothalamus, and greater IL-1 β in the hippocampus compared to pubertal males. LPS-treated adult and pubertal females show comparable central cytokine expression. Yet, adult females show greater IL-6 mRNA expression in the hippocampus compared to pubertal females. These findings highlight important age difference in the response to pathogen-induced sickness between puberty and adulthood.

Our results also show sex differences in peripheral and central immune responses following LPS treatment in mice pair-housed with an age-matched mouse. Adult females display reduced IL-6 and IL-12 concentration in the serum and IL-6 mRNA expression in the hypothalamus and IL-1 β , TNF α and IL-6 mRNA expression in the PFC compared to adult males. Interestingly, this sex difference is reversed in pubertal mice, such that, LPS-treated pubertal females display more TNF α and IL-6 mRNA expressions in the PFC and IL-1 β mRNA expression in the hypothalamus. Recent findings from our laboratory agree with these results and show that pubertal females show greater central neuroinflammatory responses to LPS compared to pubertal males (Murray et al., 2019a).

We found that age differences in LPS-induced immune responses were mitigated following microbial exchange between pubertal and adult mice. In adult males colonized with the microbes from a pubertal male, there was no difference in IL-12 and IL-6 concentration. LPS-treated pubertal males colonized with microbes from an adult mouse show a significant increase in TNF α concentration in the serum. This suggests that microbial transfer from adult males to pubertal males leads to a greater peripheral pro-inflammatory response in pubertal males. Previous findings from our laboratory show that pubertal male mice are hyporesponsive to an immune challenge, which may be maladaptive and render them more vulnerable to

enduring neural and behavioural alterations in adulthood (Girard-Joyal & Ismail, 2015). Whether increased inflammatory responses in pubertal male mice is beneficial to long-term outcomes on the brain and behaviour remains to be investigated. In females, we observed fewer age differences in peripheral cytokine concentrations and therefore, did not expect significant changes in circulating cytokines concentrations following microbial exchange between pubertal and adult female mice. Nevertheless, age differences in IFN γ and IL-10 concentrations were eliminated following microbial exchange between adult and pubertal females.

We found that microbial exchange between pubertal and adult mice also mitigates age and sex differences in LPS-induced cytokine mRNA expression in specific brain regions. LPS-treated adult males colonized with microbes from a pubertal mouse display reduced IL-1 β , TNF α and IL-6 mRNA expressions in the PFC, reduced IL-6 mRNA expression in the hypothalamus and reduced IL-1 β mRNA expression in the hippocampus compared to adult males colonized with microbes from another adult. Similarly, adult females colonized with microbes from a pubertal mouse display a decrease in IL-1 β mRNA expression in the hippocampus. In contrast, pubertal males colonized with microbes from adult males show greater IL-1 β mRNA expression in the hypothalamus and hippocampus in response to the LPS treatment. Pubertal females colonized with microbes from an adult female also show greater IL-6 mRNA expression in the PFC following LPS treatment. The ability of these microbes to alter central immune response in the host could be due to a number of factors. One possibility could be due to changes in microglial morphology and activity (i.e. neuroglia which act as the main immune defense within the brain). For example, germ-free (GF; lacking microorganisms) mice display significantly more immature microglial cell morphology compared to wild-type mice. However, microbial colonization with microbes from a wild-type mouse restores microglial cell morphology in GF

mice (Erny et al., 2017). A number of signals produced by gut microbes activate neurons within the CNS. Microbial by-products, including SCFAs and chemotactic peptides, bind to receptors on enteroendocrine cells in the GI tract and induce secretion of metabolic peptides (Lyte, 2014; Sampson & Mazmanian, 2015). These signals are relayed to the brain, via the enteric nervous system, which then activate neuronal circuits and prime essential immune cells in the brain, such as microglia. Taken together, microbial colonization likely altered production of these microbial by-products which had downstream effects on the immune system, both peripherally and within the CNS.

The current study is ongoing as we have not yet elucidated the specific microbial species that are exchanged during pair-housing. Therefore, it is unclear which microbes are priming the immune response. Nevertheless, other researchers have observed that pair-housing mice with mice of different strains with unique gut microbial compositions results in selected convergence of the gut microbiota (Campbell et al., 2012). Based on previous findings from our laboratory, pubertal CD1 mice, compared to their adult counterparts, display greater abundance of Bacteroidetes and Actinobacteria, which contain a number of bacteria that are beneficial to overall health (Murray et al., 2019b). There is a notable decline in genera within these phyla associated with aging, including protective anaerobes such as *Bifidobacteria* and *Bacteriodes* (Hopkins et al., 2001; Woodmansey, 2007). Moreover, *Clostridium* and *Bacteroides* species can induce the expansion of regulatory T cells and mitigate intestinal inflammation (Atarashi et al., 2013). Replenishment of gut with these beneficial bacteria from pubertal mice to adult mice may have regulated immune functions by strengthening the intestinal barrier, reducing intestinal permeability and reducing the inflammatory response to LPS.

We also observed changes in pubertal onset, as pair-housing with adult mice accelerated scrotal growth and vaginal opening in pubertal mice. These findings suggest that microbial transfer from adults is capable of altering gonadal steroid hormone production. The gut microbiota is believed to alter steroid hormone production through a number of mechanisms. For example, microbes can encode enzymes that regulate the balance between active and inactive steroids (Kisiela et al., 2012) and can perform oxidative, reductive and hydrolytic reactions of androgens and oestrogens (Fernandes et al., 2003; Lombardi et al., 1978). Others have demonstrated that microbial transfer from adult male mice to pubertal female mice masculinize gut microbiome composition and elevate testosterone levels in the pubertal females (Gomez et al., 2012). Therefore, a potential microbial shift in the pubertal mice may have led to accelerated pubertal development through changes in gonadal steroid hormone concentrations which may, in turn, also affect the immune response.

5. Conclusion

Our work shows that the age and sex differences in LPS-induced immune responses are modulated by the gut microbiota. Microbial transfer from pubertal mice to adult mice reduces peripheral and central inflammation following LPS treatment. Yet, these effects are more pronounced in adult males than in adult females. Following microbial transfer from adult mice, pubertal female and male mice show greater cytokine mRNA expression in specific brain regions. These results begin to provide mechanistic insight into microbial influences on the immune response. Alterations in the immune system and gut microbiome directly contribute to a number of disease states. Therefore, the gut microbiome is an important target against age and sex-specific disease risks and it is essential that we continue to expand our understanding of how gut microbiota can alter age- and sex-specific immune responses.

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COMPETING FINANCIAL INTEREST

The authors declare no competing financial interests or potential conflicts of interest.

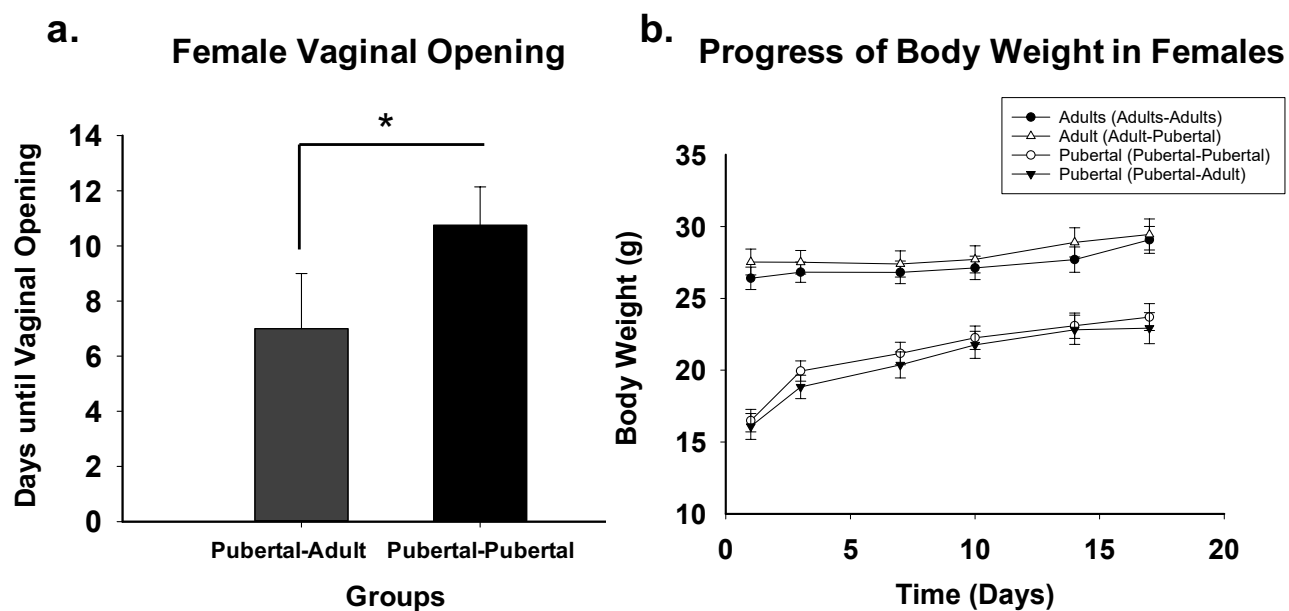


Figure 1. Mean ($\pm$ SEM) days until vaginal opening (a) and progress in body weight (b) in pubertal females pair-housed with an adult (pubertal-adult) or another pubertal (pubertal-pubertal). The asterisk (*) denote a significant difference between experimental groups ($p < 0.05$).

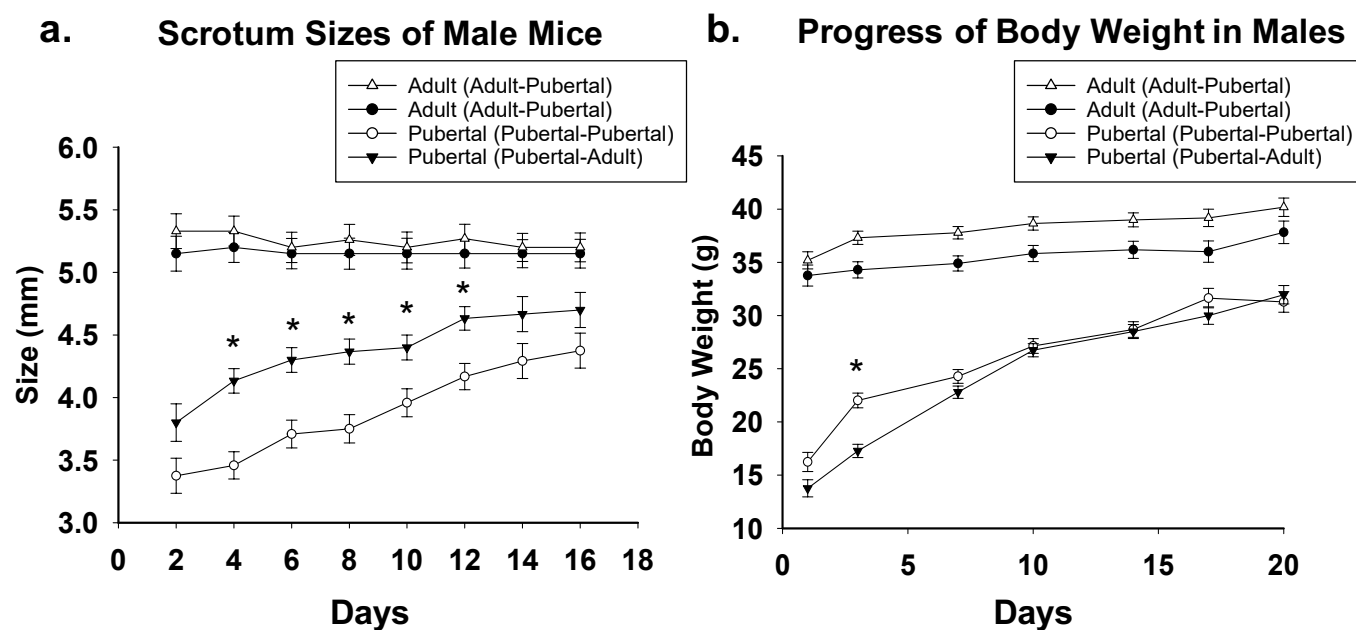


Figure 2. Mean ($\pm$ SEM) scrotum width (mm) (a) and body weight (b) recorded in pair-housed pubertal males (pubertal-pubertal or pubertal-adult) and adult males (adult-adult or adult-pubertal) over a period of 20 days. The asterisk (*) denote a significant difference in the sizes between counter-aged and identical aged experimental groups ($p < 0.05$).

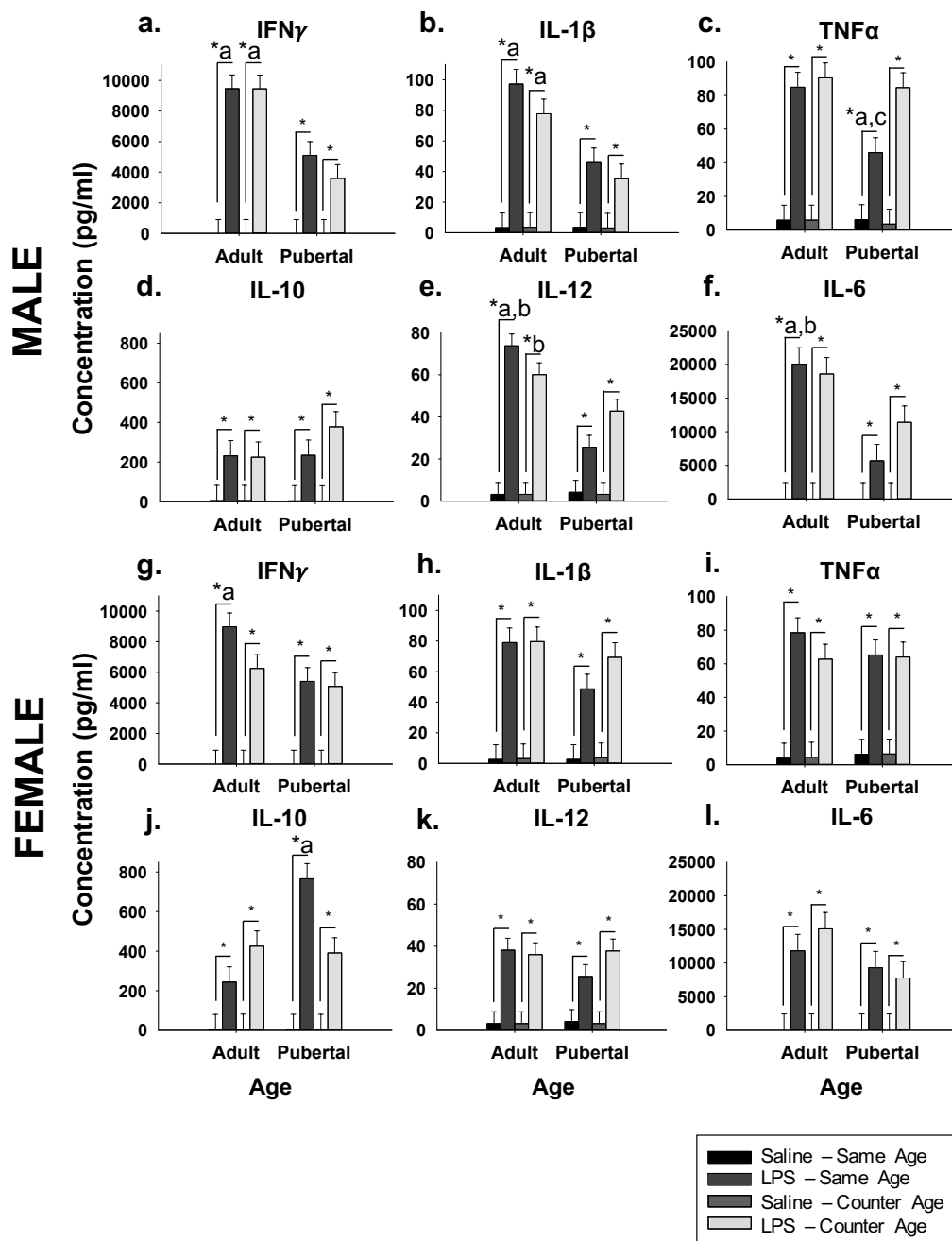


Figure 3. Pro- and anti-inflammatory serum cytokine concentrations (pg/mL) from blood in male (a-f) and female (g-l) mice eight hours following saline or LPS treatment and following a three-week-course of microbial exchange through pair-housing with an aged-matched cage mate (Saline-Same Age or LPS-Same Age) or adult and pubertal mice pair-housed together (Saline-Counter Age or LPS-Counter age). Data represented as mean ($\pm$ SEM); * $p < 0.05$, 'a' denotes a significant age difference ($p < 0.05$), 'b' denotes a significant difference between males and females, 'c' denotes a significant difference between mice pair-housed with an aged-matched cage mate or a mouse of the opposite age, $n = 10$ /group.

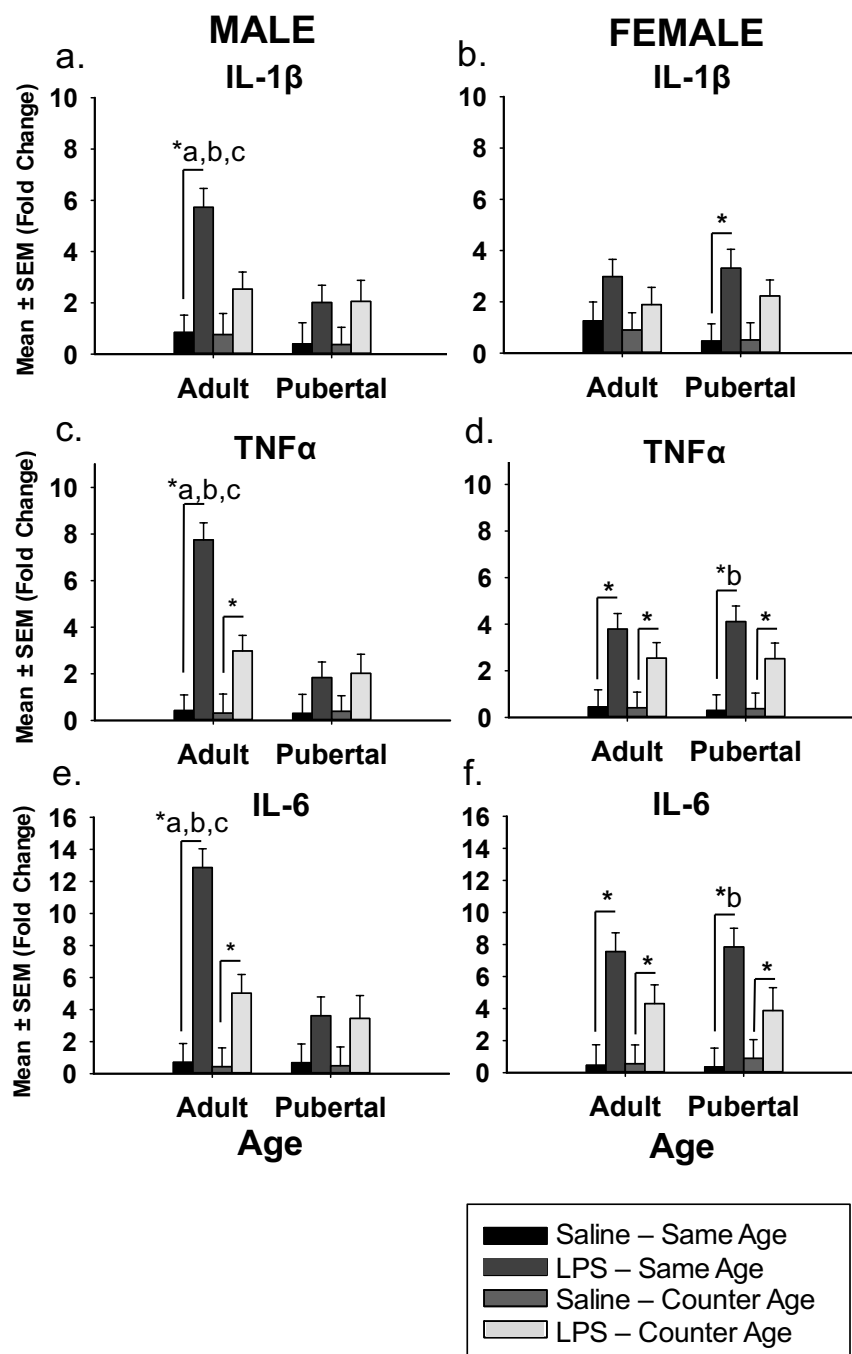


Figure 4. Cytokine mRNA expression (fold change) of IL-6, IL-1 β and TNF α mRNA in the prefrontal cortex (PFC) in male (a,c,e) and female (b,d,f) mice eight hours following saline or LPS treatment and following a three-week course of microbial exchange through pair-housing with an aged-matched cage mate (Saline-Same Age or LPS-Same Age) or adult and pubertal mice pair-housed together (Saline-Counter Age or LPS-Counter age). Data represented as mean ($\pm$ SEM); * $p < 0.05$, 'a' denotes a significant age difference ($p < 0.05$), 'b' denotes a significant difference between males and females, 'c' denotes a significant difference between mice pair-housed with an aged-matched cage mate or a mouse of the opposite age, $n = 6$ /group.

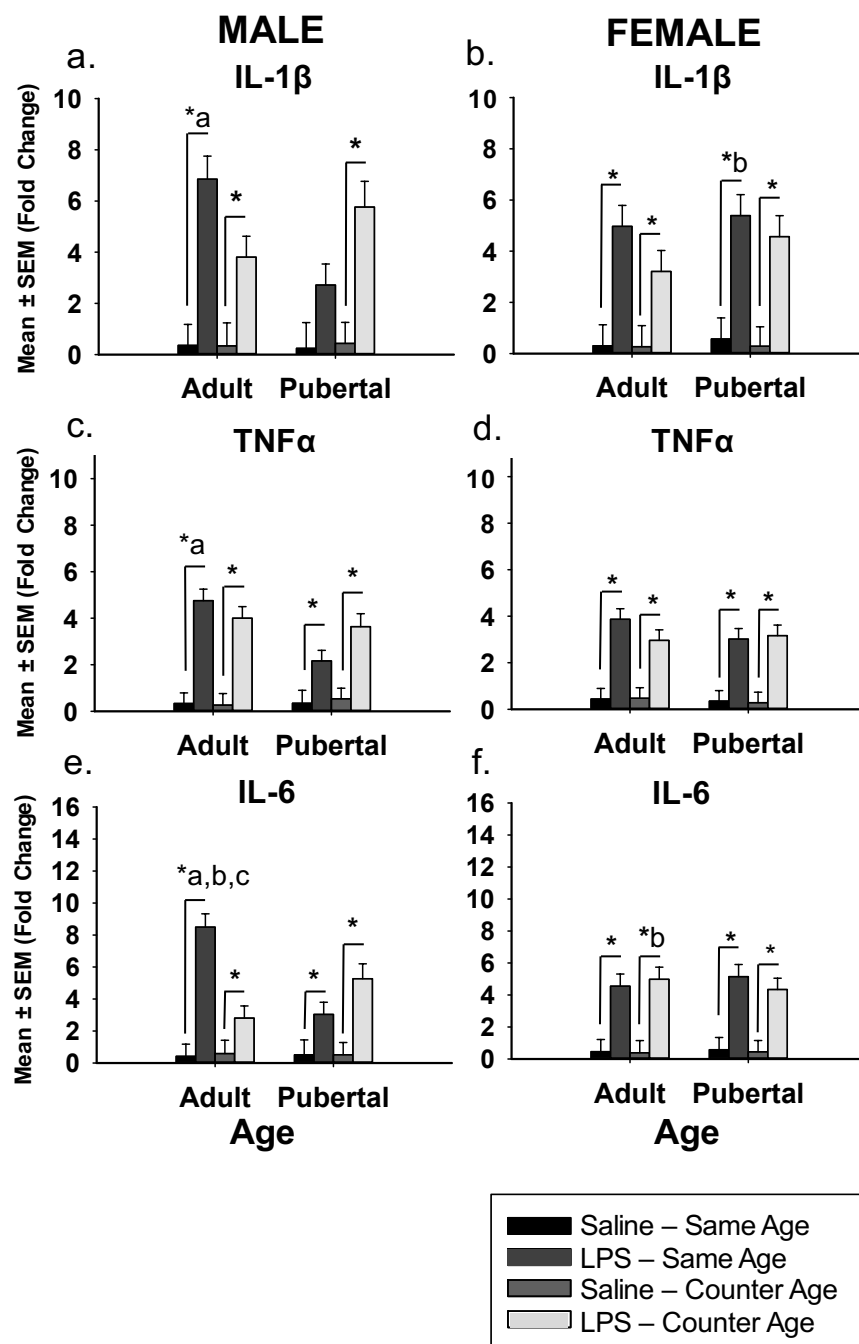


Figure 5. Cytokine mRNA expression (fold change) of IL-6, IL-1 β and TNF α mRNA in the hypothalamus in male (a,c,e) and female (b,d,f) mice eight hours following saline or LPS treatment and following a three-week course of microbial exchange through pair-housing with an aged-matched cage mate (Saline-Same Age or LPS-Same Age) or adult and pubertal mice pair-housed together (Saline-Counter Age or LPS-Counter age). Data represented as mean ($\pm$ SEM); * $p < 0.05$, 'a' denotes a significant age difference ($p < 0.05$), 'b' denotes a significant difference between males and females, 'c' denotes a significant difference between mice pair-housed with an aged-matched cage mate or a mouse of the opposite age, $n = 6$ /group.

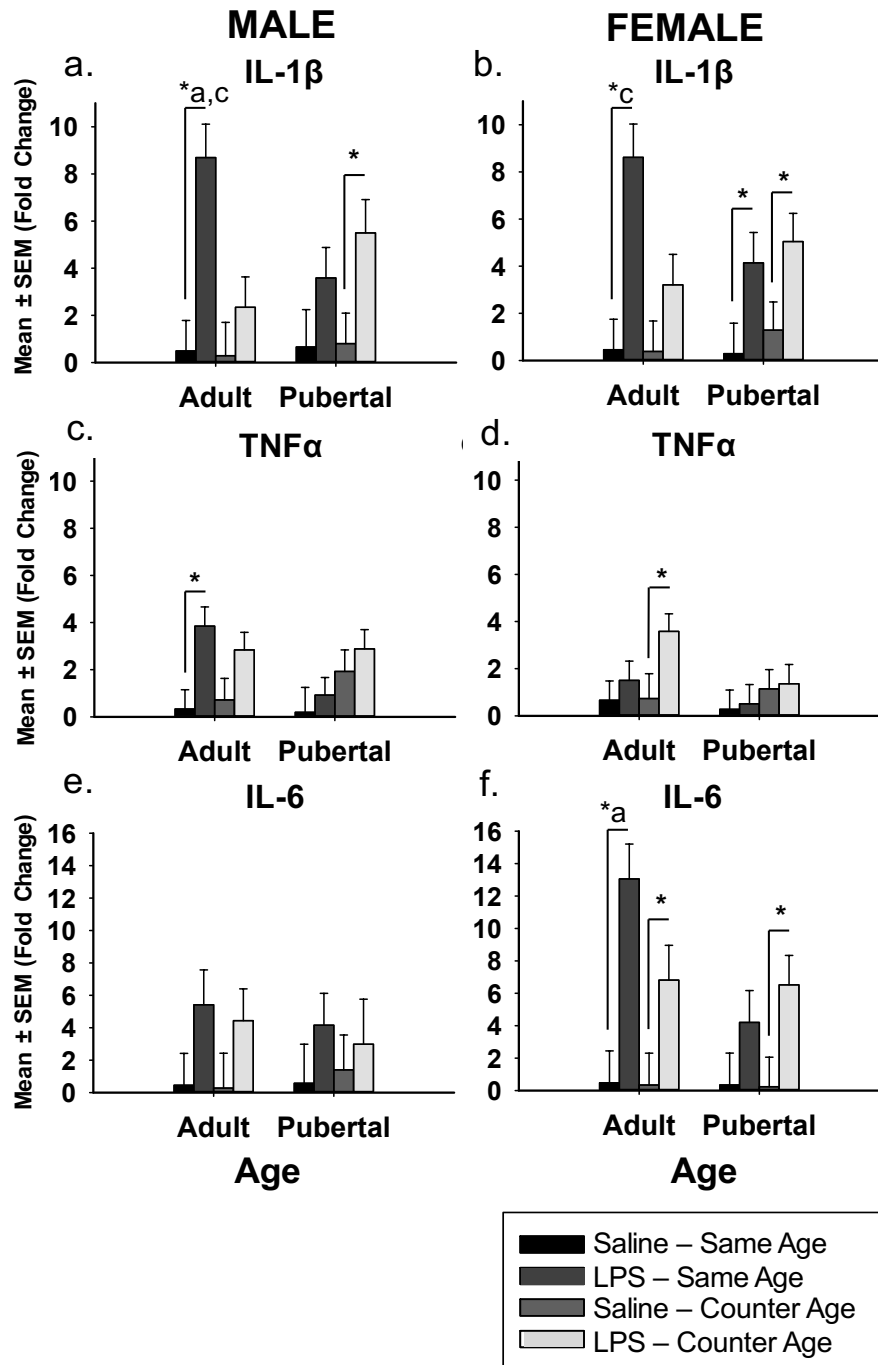


Figure 6. Cytokine mRNA expression (fold change) of IL-6, IL-1 β and TNF α mRNA in the hippocampus in male (a,c,e) and female (b,d,f) mice eight hours following saline or LPS treatment and following a three-week course of microbial exchange through pair-housing with an aged-matched cage mate (Saline-Same Age or LPS-Same Age) or adult and pubertal mice pair-housed together (Saline-Counter Age or LPS-Counter age). Data represented as mean ($\pm$ SEM); * $p < 0.05$, 'a' denotes a significant age difference ($p < 0.05$), 'b' denotes a significant difference between males and females, 'c' denotes a significant difference between mice pair-housed with an aged-matched cage mate or a mouse of the opposite age, $n = 6$ /group.

Discussion

Pubertal exposure to an immune challenge (lipopolysaccharide; LPS) causes lasting changes in reproductive and non-reproductive behaviors, like cognitive functioning, depression- and anxiety-like behaviors in mice (Ismail and Blaustein, 2013; Ismail et al., 2013; Ismail et al., 2011; Laroche et al., 2009a; Laroche et al., 2009b; Olesen et al., 2011). However, the mechanisms underlying these enduring effects are unknown. We speculated that one possible mediator was the gut microbiome, as it communicates bi-directionally with the brain and the immune system.

The gut microbiome is inhabited by trillions of microorganisms, which are estimated to be more than 10 times greater than the number of cells in the human body (Gill et al., 2006; Qin et al., 2010). These microorganisms are known to promote and regulate the immune system, regulate steroid hormone activity and shape brain development (Belkaid & Hand, 2014; Bercik et al., 2010; Crumeyrolle-Arias et al., 2014; Fung et al., 2017; Ho & Ross, 2017; Jasarevic et al., 2015; Kennedy et al., 2016). Gut microbes promote and regulate immune function largely by stimulating gut epithelial cells (e.g. Toll-like receptors; TLRs), gut dendritic or T regulatory cells and triggering the production of pro- and anti-inflammatory cytokines (Belkaid & Hand, 2014). The gut communicates with the brain through sympathetic and parasympathetic branches of the autonomic nervous system (ANS), the enteric nervous system (ENS), and neuroendocrine and neuroimmune pathways (Browning and Travagli, 2014; Cryan & Dinan, 2011; Foster et al., 2017; Grenham et al., 2011). This bi-directional interplay between the gut and the brain has been termed the “gut-brain axis”. Despite our growing understanding of gut-brain axis functioning, there is remarkably limited research examining its role in brain development and maturation during critical periods, such as puberty.

Given this important knowledge gap, the current studies were carried out to advance our understanding of the role of the gut microbiome during puberty on neural and behavioral outcomes. These studies have addressed the following research questions: 1) Are there age- and sex-related differences in gut microbial composition? 2) Does exposure to an immune challenge alter the gut microbiota in an age and sex-specific manner? 3) Can gut microbial transfer between pubertal and adult mice alter age- and sex-specific LPS-induced immune responses? 4) Does pubertal exposure to probiotics alter the acute immune response to LPS treatment in males and females? 5) Does pubertal exposure to probiotics prevent enduring LPS-induced outcomes on cognition, depression-like and anxiety-like behaviors in adulthood?

Gut microbial composition is age-dependent

The gut microbiome changes rapidly during infancy, early childhood and puberty/adolescence, and stabilizes in adulthood (Hollister et al., 2015; Jasarevic et al., 2015). Our findings demonstrate an age difference in microbial composition between pubertal and adult male and female mice (Murray et al., 2019b). Specifically, adult male and female mice display less Bacteroidetes phylum compared to pubertal mice. Adult female mice also display significantly greater Firmicutes compared to their pubertal counterparts (Murray et al., 2019b). These results are consistent with previously published findings showing that Firmicutes increase in abundance in the gut microbiome with age (Mariat et al., 2009). Bacteroidetes and Firmicutes are the dominant bacterial phylotypes in the gut microbiome, while Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia phyla are expressed in lower abundance (Eckburg et al., 2005). Species of bacteria within the Firmicutes phylum are largely gram-positive, while Bacteroidetes are gram-negative. These phyla contain diverse species of bacteria with numerous functional capacities (Wexler & Goodman, 2017). For example, the prominent

genus within the Bacteroidetes phylum, *Bacteroides*, can synthesize conjugated linoleic acid, which is known to be antidiabetic, and exert other immunomodulatory properties (Devillard et al., 2009), suggesting that reduced abundance of Bacteroidetes is detrimental to host immune function. Moreover, the decrease in the Bacteroidetes: Firmicutes ratio is linked to poorer defense against pathogens and increased inflammatory response (Mariat et al., 2009; Kim et al., 2016). Our findings show that adult mice display greater inflammatory responses following LPS treatment compared to pubertal mice (Paper 3), suggesting that age differences in microbial composition prime age differences in the immune response.

The current thesis found no sex difference in gut microbial composition during puberty when analyzed at the phylum level (Murray et al., 2019a). While some studies have found limited effects of sex on the gut microbiota (Huttenhower, 2012; Kovacs et al., 2011), others have demonstrated unique sex differences in microbial composition (Bolnick et al., 2014; Shastri et al., 2015). For example, a time-series study characterized the gut microbial composition in mice before puberty, after puberty, and in adulthood, and observed that sex differences in microbial composition only emerged after puberty (Yurkoveskiy et al., 2013). Interestingly, Yurkoveskiy and colleagues (2013) observed that male mice show greater microbial changes across the lifespan, while microbial composition of adult females remains similar to that of pre-pubertal mice. However, castration prior to puberty eliminated sex differences in gut microbiota composition in adult males. These findings and others suggest that sex differences in microbial composition are primed by gonadal hormones during pubertal and adolescent development (Hollister et al., 2015; Ober et al., 2008). The direct mechanisms through which hormones mediate microbial composition are largely unknown. Given that gonadal steroid hormone concentrations begin to rise during puberty and adolescence and peak in early adulthood

(Worthman & Stallings, 1997), it is possible that gonadal hormone concentrations at 6 weeks old have not yet reached the threshold to induce hormone-mediated alterations in gut microbiota.

The current findings reveal a sex difference in microbial composition after one week of probiotic treatment (Murray et al., 2019a). Specifically, probiotic consumption induced greater microbial changes in pubertal males than in pubertal females. Probiotics are known to alter the microbial environment in a number of ways. For example, probiotics compete for receptors with other intestinal microbes (Collado et al., 2007), or produce antimicrobial agents which suppress the growth of other microorganisms (O'Shea et al., 2011). Studies examining sex-specific microbial changes following probiotic treatment are limited. However, Suzuki and colleagues (2017) have observed that the frequency of probiotic consumption correlates negatively with the presence of Enterobacteriaceae and Staphylococcus in males, but not in females (Suzuki et al., 2017). Moreover, 45 days of probiotic consumption causes an increase in *Staphylococcus* and *Roseburia* in female mice, but an increase in short-chain fatty acids (SCFAs; i.e. butyrate and acetate) in males (Karunasena et al., 2014). Thus, our findings further demonstrate that there are important sex differences in the colonization of probiotic bacteria and its influence on host microbial environment.

LPS treatment alters the gut microbiome, in a sex-specific manner

Numerous factors, like pathogenic bacteria, birth mode, antibiotics, probiotics and diet, can alter the gut microbiome by structurally changing intestinal epithelial cells (IECs), mucosal lining and production and secretion of microbial by-products (Furness et al., 2013; Human Microbiome Project, 2012; Panda et al., 2014) and/or by changing gut function (Jandhyala et al., 2015; Grenham et al., 2011). Functional changes to the gut include alterations to immune signalling, production of anti-microbial factors, pathogen displacement, metabolism and vitamin

synthesis (Grenham et al., 2011; Hooper et al., 2001; Cario et al., 2004; Vaishnava et al., 2008). Lipopolysaccharide, a major component of the outer layer of gram-negative bacteria, is one of the most potent activators of innate immune signaling. Intestinal epithelial cells (IECs) in the gut lumen recognize LPS through toll-like receptor 4 (TLR4) binding, which leads to the release of proinflammatory cytokines (Lotz et al., 2006; Lotz et al., 2007). The current thesis demonstrates that LPS treatment significantly disrupts gut microbial composition of pubertal mice relative to adult mice (Murray et al., 2019b). Following LPS treatment, pubertal males show an increase in Proteobacteria but a decrease in Bacteroidetes, while pubertal females display a decrease in Firmicutes, compared to saline-injected controls (Murray et al., 2019a). Proteobacteria contain a wide variety of gram-negative pathogenic bacteria such as *Escherichia* and *Salmonella* (Rizzatti et al., 2017). Increases in a number of bacteria within the Proteobacteria phylum are observed in animal models of inflammation related to irritable bowel disease (Lupp et al., 2007), suggesting that this phylum is linked to disease states and that inflammation-induced increases in Proteobacteria is not only caused by LPS but also by other inflammation inducing factors. An analysis of bacterial genera, a lower taxonomic classification rank than phyla, demonstrated that LPS treatment causes greater bacterial depletion in pubertal males compared to pubertal females (Murray et al., 2019b). While the mechanisms for these sex-specific microbial responses to an immune challenge are unclear, studies in rodents have demonstrated that males display greater susceptibility to a number of parasites (protozoa, helminth, cestodes and arthropods), fungi, bacteria and viruses compared to females (Bernin et al., 2014; Daniels, 1994; Lunzen et al., 2014; Sano, 1992). This increased susceptibility is thought to be due to lower innate immune responses in males compared to females (Ahmed et al., 2010; Cai et al., 2016; Klein, 2000; Rettew et al., 2010). Females generally show greater antigen presenting activity, higher

immunoglobulin levels and more antibody production than males (Fish, 2008). Females are also more resistance to gut injury compared to male counterparts (Homma et al., 2005). Therefore, it is likely that our males experience greater LPS-induced gut dysbiosis due to sex differences in immunity.

Microbiota transfer between adult and pubertal mice mitigates age and sex differences in the immune response

Our findings demonstrate that microbial transfer between adult and pubertal male and female mice mitigates age and sex differences in LPS-induced inflammatory responses (Paper 3). Following LPS treatment, pubertal males and females, that are not exposed to microbial transfer from adult mice, display lower cytokine concentrations in serum and lower cytokine mRNA expression in the brain compared to adult counterparts. However, adult mice colonized with microbes from pubertal mice display reduced peripheral and central LPS-induced inflammatory responses compared to adult that were not colonized with pubertal microbes. Moreover, pubertal mice colonized with microbes from adult mice display slight increases in LPS-induced inflammation. This study is ongoing to identify which gut bacteria are transferred between adult and pubertal mice and alter the immune response. Our findings also show that pubertal mice display greater microbial diversity and greater abundance of beneficial bacterial genera including *Lactobacillus* and *Bifidobacterium* compared to adults (Murray et al., 2019b). Both *Lactobacillus* and *Bifidobacterium* have been shown to exhibit anti-inflammatory effects in mice (Mitsuoka, 1990; Oh et al., 2018). There are a number of mechanisms through which these bacteria exert anti-inflammatory functions. For example, they both produce SCFAs, which activate receptors on intestinal epithelial cells to inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway, resulting in less inflammation (Liu et al., 2018). Since pubertal mice

expressed greater abundance of the *Lactobacillus* and *Bifidobacterium* genera (Murray et al., 2019b), the transfer of these beneficial microbes from pubertal to adult mice could have protective effects on the response to infection.

The direct mechanisms through which species of microbes alter the microbial environment and program central immune responses require further investigation. One possibility is through microglial priming, an essential immune cell in the brain. Interestingly, germ-free (GF) mice (lacking microorganisms in the gut) display greater numbers of immature microglia compared to conventionally colonized controls. However, housing GF mice with conventional colonized controls, promotes microglia maturation in GF mice (Erny et al., 2015). Moreover, it is speculated that microglia maturation is facilitated by microbial-derived SCFAs, which can restore microglial abnormalities (Borre et al., 2014; Erny et al., 2015). Microglia can change in structure and function throughout the lifespan (Lourbopoulos et al., 2015; Tay et al., 2016). Therefore, it is possible that adult mice colonized with beneficial microbes from pubertal mice display a greater number of mature microglia in the CNS, which could lessen the central inflammatory response. Taken together, our findings and others highlight that gut microbiota have far-reaching effects on the immune system both peripherally and in the brain.

Pubertal mice show increased immune responses following microbial colonization from adult counterparts (Paper 3), which could be due to a number of factors. One possibility is that adult mice may transfer more gram-negative bacteria to pubertal mice, resulting in greater inflammation and dysbiosis in the microbial environment. We also noted that microbial colonization accelerated pubertal development in both sexes, suggesting that microbial and hormonal exchanges from adult mice can alter pubertal maturation. Whether microbial

colonization from adult mice to pubertal mice can prevent the enduring LPS-induced behavioral alterations remains unclear.

Pubertal exposure to probiotics reduces acute immune responses to LPS treatment

We further investigated the role of the gut microbiome in moderating these age- and sex-specific immune responses with probiotics (Murray et al., 2019a; Murray et al., 2019b). Our findings show that the consumption of probiotics during puberty mitigates LPS-induced peripheral and central inflammatory response and sickness behaviour (Murray et al., 2019a). Probiotics produce bioactive compounds which directly modulate the immune system through mucosal immune cells or the epithelial barrier (Akira, 2009; Hemarajata & Versalovic, 2013) and decrease the production of pro-inflammatory cytokines (O'Mahony et al., 2005; Hemarajata & Versalovic 2013; D'Mello et al., 2015; Schachter et al., 2018). Both of our probiotic treatments (Murray et al., 2019a; Murray et al., 2019b) included species of the *Lactobacillus* genera, which have been demonstrated to enhance the intestinal barrier (Mack et al., 2003) and exert antibacterial effects on harmful bacteria (Collado et al., 2007; Servin, 2004). Although the mechanisms have not been fully elucidated, administration of probiotics can displace pathogenic bacteria by rebalancing microbial communities, reinforcing barrier function and lowering the pH of the colon (Gagliardi et al., 2018). Taken together, our findings demonstrate that probiotics significantly reduce LPS-induced inflammation both peripherally and in the brain by altering gut microbial composition.

Pubertal LPS causes sex-specific neural and behavioral alterations in adulthood

This thesis has established an animal model for the sex-specific enduring effects of an immune challenge during puberty. The findings show that pubertal LPS treatment results in increased anxiety-like behaviour and heightened stress reactivity to a novel stressor in adult male

mice (Murray et al., 2019a; Murray et al., 2019b) and increased depression-like behavior in adult female mice. Males treated with LPS during puberty also display greater TLR4 expression in the paraventricular nucleus of the hypothalamus (PVN) (Murray et al., 2019a), and greater c-Fos expression in the hippocampus (Murray et al., 2019b) following a novel stressor in adulthood. Taken together, pubertal LPS treatment causes enduring behavioural and neurochemical alterations in both sexes. However, LPS treatment may activate different mechanisms in males and females, which could explain the sex-specific outcomes in adulthood. These findings stress the importance of advancing our understanding of the mechanisms contributing to sex differences in mental health outcomes.

There are a number of reasons for these sex-specific vulnerabilities. One possibility is that pubertal LPS treatment caused greater microbial disruption, gut dysbiosis and a global inflammatory response in males which programmed TLR4 responses in the PVN later in life. Males also show transient increases in blood-brain barrier (BBB) permeability at four hours following LPS treatment, while estradiol is suggested to protect BBB integrity. Specifically, females show greater BBB permeability following ovariectomy but treatment with estradiol reverses this effect (Maggioli et al., 2016). Moreover, in response to the same immune challenge, pubertal males show greater serum pro-inflammatory cytokine concentrations than females (Murray et al., 2019a). These findings suggest an inefficient immune response to pathogens in pubertal males and may explain the lasting effects on anxiety-like behaviour and hypothalamic-pituitary-adrenal (HPA) axis responses in adulthood. Another possibility is that LPS treatment alters estrogen receptor expression in females. Previous work also demonstrates that pubertal LPS treatment in females alters the responsiveness to estradiol in adulthood in depression-like behaviour (Ismail et al., 2012). This could be due to a decrease in estrogen receptor β , which

mediate the antidepressant effects of estradiol (Osterlund, 2010), which requires further investigation. Nevertheless, the current work developed a female CD1 mouse model of pubertal stress-induced depression. Based on clinical cross-sectional research, there is a change from an equal female : male prevalence of depression prepuberty to a 2:1 female : male prevalence after puberty (Angold and Costello, 2006; Bebbington et al., 1998). This finding highlights the important link between puberty and the onset of depression in females and the need to advance our understanding of the increased vulnerability to depression in post-pubertal females. Our model can be used to further advance our understanding of the mechanisms underlying stress-induced depression and anxiety and the increased sex-specific risks for certain mental health conditions.

Probiotics prevent enduring LPS-induced depression- and anxiety-behaviour, and stress-induced brain activation in adulthood

The current dissertation demonstrates that a short exposure to probiotics during pubertal development blocks the enduring negative consequences of a pubertal immune challenge. Specifically, pubertal exposure to probiotics prevents enduring LPS-induced depression-like behaviour in females (Murray et al., 2019a) and anxiety-like behaviors in males (Murray et al., 2019a; Murray et al., 2019b). Probiotics also mitigate LPS-induced increase in TLR4 expression in the PVN (Murray et al., 2019a) and c-Fos expression in the hippocampus in response to a novel stressor in adult males (Murray et al. 2019b). We speculate that probiotics are blocking the enduring outcomes of pubertal LPS treatment through a number of mechanisms. One possibility is that pubertal probiotic treatment block the enduring effects of pubertal LPS treatment by decreasing the acute inflammatory response to LPS treatment. This is consistent with our findings as we found that our probiotic treatment reduced LPS-induced inflammation both

peripherally and in the brain. Another possibility is that probiotic treatment strengthens tight junctions (i.e. structures between adjacent epithelial cells that regulate transport of substances across the intestinal barrier) resulting in less gut permeability and inflammation (Anderson et al., 1993; Arrieta et al., 2006). It is also possible that probiotics block pubertal LPS-induced alterations in HPA axis development. Early life stress is demonstrated to disrupt both the neuroplasticity of the PVN and in the maturation of the HPA axis (Amath et al., 2012; Green et al., 2011; Van den Bergh et al., 2008). Pioneer work by Sudo and colleagues (2004) observed that gut microbes play a fundamental role in the development of the HPA axis during early critical periods of development. Specifically, GF mice display exaggerated release of adrenocorticotrophic hormone. However, this effect is fully reversed with the probiotic *Bifobacterium infantis*, but only if exposure to these microbes occur at an early stage in postnatal developmental and not later. Puberty is a second important period of HPA axis development (McCormick & Mathews, 2007; Romeo, 2005; Romeo & McEwen, 2006) and our work further demonstrates that gut microbes are critical during pubertal development against these stress-induced outcomes (i.e. heightened stress reactivity, anxiety and depression). Given that mice were not consuming probiotics at the time of behaviour testing in adulthood, our work demonstrates that a relatively short exposure to probiotics during a critical window in development, such as puberty, can prevent enduring LPS-induced behavioural outcomes.

Limitations

The current studies have certain limitations. First, we did not examine the effect of pubertal LPS treatment on the permeability of the gut, a term referred to as “leaky gut” (Foster et al., 2017; Gareau et al., 2008). It is possible that pubertal LPS treatment induced damage to the mucosal lining and resulted in an increased intestinal permeability and translocation of gram-

negative bacteria across the gut mucosal lining, which could provide a mechanism for LPS-induced neural and behavioral alterations. Second, our studies cannot explain how probiotic alters early inflammatory reactions or the recovery following LPS treatment, as we examined acute LPS-induced inflammatory responses at eight hours in both probiotic studies (Murry et al., 2019a; Murray et al., 2019b). Eight hours was selected because all LPS-treated mice are showing many sickness behaviours at this time point. Third, the current studies cannot determine if mice of other ages (pre- or post-pubertal) would also show enduring behavioural effects of probiotics into adulthood. Due to the numerous groups and manipulations, including mice of other ages was not feasible in the current research design. Lastly, our investigation of microbial colonization (Paper 3) did not include blood collection during the pair-housing in order to minimize additional handling and stress. This study cannot address if pair-housing with an older or younger mouse alters gonadal steroid hormone concentration which represents a possible limitation. We speculate that the ongoing microbial analysis will address this concern by demonstrating microbial colonization between our treatment groups.

Future Direction

Future studies should focus on identifying the mechanisms through which pubertal LPS treatment and probiotic treatment causes alterations to the gut microbiome, immune functions and enduring effects on behaviour and CNS functions in adulthood. For example, future studies could investigate whether pubertal LPS treatment alters the permeability of the gut and of the blood brain barrier (BBB). Future studies could also investigate if the effects of probiotics on immune response are mediated by changes to SCFAs (acetate, propionate and butyrate) to elucidate a potential signaling mechanism which can cause alterations to the immune system and CNS, such as alterations in microglia morphology. Future studies should attempt to identify the

specific microbes that are modulating age differences in the immune response and the long-term effects of microbial transfer from adult mice to pubertal mice on pubertal LPS-induced enduring changes in behaviour.

Future studies also should focus on the gut microbiome in complex disorders such as anxiety and depression and cellular and microbial mechanisms driving sex-specific effects. For example, it should be examined if males and females exposed to an immune challenge during puberty show chronic elevations in inflammatory cytokines or gut dysfunction into adulthood. Future studies should also examine short and long-term the efficacy of probiotics in human adolescent populations on mood and anxiety.

Summary

These current studies have filled an important knowledge gap in our understanding of the pubertal gut microbiome, stress and immune responses, and on mechanisms contributing to the development of anxiety and depression. This dissertation has established that there are important age differences in gut microbial composition and age and sex differences in gut microbial composition following an immune challenge. Altering the gut microbiome, through probiotics or microbial transfer between adult and pubertal mice, changes the peripheral and central inflammatory responses to an immune challenge. These findings highlight the significant regulatory influence of gut microbiota against immune challenges. We also demonstrated that exposure to an immune challenge during puberty causes enduring sex-specific behavioural and neurochemical alterations. However, manipulation of the gut microbiome with probiotics during puberty blocks these LPS-induced behavioral and neurochemical alterations. The findings of the current dissertation add to our understanding of sex-specific pubertal vulnerabilities to immune challenge and the central role of the gut microbiome on neurobehavioral outcomes later in life. It

is critical that we continue to investigate the link between the gut microbiota and mental health and find innovative approaches to protect the brain from certain stressors during critical periods of development and prevent the onset of stress-related mental health conditions.

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