THE EFFECT OF MICROBIAL COMPOSITION ON ACUTE IMMUNE RESPONSIVITY IN PUBERTAL MALE AND FEMALE CD1 MICE

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Table of Contents

List of Figures

List of Abbreviations

ACTH	Adrenocorticotropic hormone
ATP	Adenosine tri-phosphate
AMPs	Anti-microbial patterns
AMNS	Ampicillin, metrodinazole, neomycin, streptomycin
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
CD14	Cluster of differentiation 14
CNS	Central nervous system
CTL	Control
CVOs	Circumventricular organs
EDTA	Ethylenediaminetetraacetic acid
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GRs	Glucocorticoid receptors
GREs	Glucocorticoid response elements
HPA	Hypothalamic-pituitary-gonadal axis
HPG	Hypothalamic-pituitary-gonadal axis
Ig	immunoglobulin
ILs	Interleukins
JNK	c-Jun N-terminal kinase
LBP	Lipopolysaccharide binding protein
LH	Luteinizing hormone

LPS	Lipopolysaccharide
MAMP	Microbe-associated molecular pattern
MPOA	Medial preoptic area
MRs	Mineralocorticoid receptors
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
NF- κβ	Nuclear transcription factor kappa beta
NK	Natural killer
NLRP3	Nucleotide-binding domain family pyrin domain containing 3
NMDA	N-Methyl d-aspartic acid
NO	Nitric Oxide
NOS	Nitrogen oxidative species
PGE	Prostaglandin
PVN	Paraventricular nucleus
ROS	Reactive oxidative species
Rt-qPCR	Real-time quantitative polymerase chain reaction
SAL	Saline
SCFAs	Short chain fatty acids
TNF-α	Tumour necrosis factor alpha
ZO-1	Zonula occludens-1

Abstract

The gut microbiome plays a quintessential role in the development and maintenance of the neuro-immune system throughout lifespan. Changes in microbial composition during critical periods of development—such as puberty—are associated with long-term disruptions in brain function and neuro-immune responsivity. However, the mechanisms underlying the effect of microbial dysbiosis on the pubertal neuro-immune response has yet to be elucidated. Therefore, the current thesis was designed to investigate the effect of changes in gut microbial composition (through antibiotic and probiotic exposure) in pubertal male and female mice on acute lipopolysaccharide (LPS)-induced immune response. Male and female CD1 mice were treated with one week of antibiotic treatment (mixed antibiotics or water) and probiotic treatment (lacidofil, probio'stick, or placebo) at five weeks of age. At six weeks of age (pubertal stress-sensitive period), the mice received a single injection of LPS or saline. Sickness behaviours were assessed and mice were euthanized at eight hours' post-injection. Brain, blood, and intestinal measures were collected. The results indicated that the antibiotic treatment reduced sickness behaviours, increased LPS-induced plasma cytokine concentrations, and LPS-induced hippocampal pro-inflammatory markers in a sexually dimorphic manner. Probiotics reduced LPS-induced plasma cytokine concentrations and hippocampal pro-inflammatory markers in a sexually dimorphic manner. Lacidofil supplementation mitigated antibiotic-induced plasma cytokine concentrations and sickness behaviours. These findings suggest that the microbiome is an important modulator of the pro-inflammatory immune response during puberty. These results also have implications for the neuro-immune mechanisms with which gut dysfunctions influence brain function and behaviours.

Statement of Problem

Puberty is a critical period of development, marked by significant neural reorganization in the brain. During this period, microglia aid in the maturation of the pubertal neural network through synaptic pruning, neuronal proliferation and differentiation, as well as phagocytic functions (see review: Schafer et al., 2012). Exposure to an immune stressor (e.g. lipopolysaccharide) during this period can cause long-term reductions in neurogenesis, and synaptic connectivity in the hippocampus (Valero et al., 2014). The gut microbiome is a key regulator of neuro-immune responsivity; alterations in microbial composition impact how microglia respond to a pathogenic exposure. For example, supplementation with kefir (a milk-based probiotic) prior to pubertal onset significantly reduces central cytokine expression after lipopolysaccharide (LPS) exposure (Murray et al., 2019). As well, microbial suppression (through antibiotic consumption) in mice is associated with increased neuro-inflammation and oxidative stress, and immature microglial morphologies (Erny et al., 2015). Supplementation with probiotics (e.g. *Lactobacilli* strains) reverses these effects (D'Mello et al., 2015). As such, gut microbial composition influences microglial morphology and function, and can mitigate or exacerbate neuro-inflammation in the brain. Microbiome research assessing neuro-immune responsivity is in its early stages, yet promising. However, the current literature on microbial dysbiosis focuses on germ-free models or antibiotic-treated adult mice. Little is known about the mechanism with which the microbiome influences neuro-immune responsivity, and how acute microbial shifts during developmental periods—such as puberty—impact the acute immune response. Therefore, our research will investigate how changes in the gut microbiota (through antibiotic and probiotic supplementation) in pubertal male and female CD-1 mice impacts neuro-

immune signalling after exposure to lipopolysaccharide. To begin, this paper will provide the background and rationale for the thesis question.

Conceptual and Theoretical Background

Pubertal development

Throughout the lifespan, there are multiple periods of development that are sensitive to immune challenges. Challenges during these critical periods can have short and long-term physiological and psychological consequences. One of these critical periods is puberty; defined as a physiological maturation of reproductive systems into an adult-like phenotype (reviewed in Sisk & Foster, 2004). Pubertal onset begins with an increase in the firing rate of gonadotropin releasing hormone (GnRH) secreting neurons in the hypothalamus, a component of the hypothalamic-pituitary-gonadal (HPG) axis (Herbison, 2016). Increased secretion of GnRH into the hypophysial portal veins of the anterior pituitary induces secretion of gonadotropins (i.e. luteinizing hormone [LH] and follicle stimulating hormone [FSH]) into the bloodstream. LH and FSH bind to their receptors in the gonads and induce maturation of gametes (gametogenesis), and the production of gonadal steroid hormones, such as androgens, estrogens, and progestins (Ebling & Cronin, 2000). The increased activity of the HPG axis and circulating gonadal hormones are involved in the development of secondary sexual characteristics, maturation of sexual behaviours, and sexually dimorphic neural reorganization (Foster et al., 2002). This includes synaptic remodelling (Feinberg, 1982), cell proliferation (Tanapat et al., 1999), and apoptosis (Nuñez et al., 2001). Microglia aid in the maturation of the pubertal neural network through synaptic pruning, neuronal proliferation and differentiation, and phagocytic functions (see review: Schafer et al., 2012). Once an adult phenotype is obtained, microglia transition to

actively monitoring the neural network (Abdel-Haq et al., 2019). Thus, pubertal neural reorganization is permanent and limited to early development. Due to the active and non-homeostatic state of the pubescent brain, exposure to stress during pubertal development can have long-term effects on brain function and behaviours (Kane & Ismail, 2017).

Consequently, puberty is a period of development where the brain is sensitive to neuro-endocrine dysregulation. A single dose of lipopolysaccharide (LPS; 1.5 mg/kg) during puberty (6 weeks of age) in CD-1 mice suppressed sexual receptivity in female adults (Ismail et al., 2011). Further exploration has shown that other stressors (i.e. shipping stress) at six weeks of age was associated with the down-regulation of ER-α expression in the medial preoptic area, ventromedial nucleus, and the arcuate nucleus of the hypothalamus (Ismail et al., 2011). Similar research has shown that LPS exposure at 6 weeks of age induces cognitive deficits, increased anxiety in males, and increased depressive-like behaviours in females in adulthood (Cai et al., 2016; Kolmogorova et al., 2019). The results suggest that immune stressors during puberty impair reproductive and non-reproductive behaviours in adulthood through physiological changes in brain structure and function (Kane & Ismail, 2017). It has been theorized that the neural circuitry of pubertal mice is more susceptible to damage through the upregulation of pro-inflammatory markers during an acute immune response.

The acute immune response

The immune system's role is to protect an organism from invading pathogens. There are two components to this system, the acute and adaptive responses, which work in concert to eliminate pathogens. The acute response recognizes pathogens through pathogen associated molecular patterns (PAMPs), which are small carbohydrate structures located on a pathogen's membrane.

PAMPs bind to receptors on immune cells and are recognized as non-self-carbohydrates by immune cells. For example, lipopolysaccharide (LPS) is a PAMP found in the cellular membrane of gram-negative bacteria, and is used extensively in animal research to initiate an acute immune reaction (Cai et al., 2016).

LPS is a highly-ordered structure containing heteropolysaccharides (De Castro et al., 2010), which binds to toll-like receptors (TLRs) on innate immune cells (Lehnardt, 2010). Specifically, LPS binds to lipopolysaccharide binding protein (LBP), forming an LPS-LBP complex in the TLR receptor. This complex is transferred to a membrane-anchored protein (CD14), solely responsible for LPS recognition. From there, CD14 and the LPS-LBP complexes associate with TLR4 and lymphocyte antigen 96 (MD-2), forming a tri-molecular receptor cluster that induces intracellular signalling (reviewed in Triantafilou & Triantafilou, 2005).

After homo-dimerization of the MD-2/TLR-4 complex, two signalling pathways are induced, early MyD88 signalling, and late MyD88-independent signalling. MyD88 pathway activation leads to the translocation of nuclear transcription factor kappa beta (NF- κβ), activator proteins, and interferon regulatory factors into the nucleus of the immune cell (reviewed in Pålsson-McDermott & O'Neill, 2004). NF- κβ upregulates cytokines (pro-inflammatory and anti-inflammatory molecules) such as tumour necrosis factors (TNF-α) and interleukin factors (ILs; Parkin & Cohen, 2001). As well, NF- κβ is involved in promoting transcription of immune-receptors, chemokines, cyclo-oxygenase, and NO synthase (Dantzer, 2004). It is common for researchers to use pro-inflammatory and anti-inflammatory cytokines as measures of immune responsivity (e.g. the strength of the immune response). This includes (but is not limited to) interleukin factors (e.g. IL-6, IL-1B), interferons, and tumour necrosis factors (TNF-α).

These immune factors all have a role in organizing the acute immune response to an invading pathogen. For example, circulating pro-inflammatory cytokines converge on the brain to induce symptoms such as lethargy, huddling, pilo-erection, and changes in body temperature. Non-specific symptoms exhibited after pathogen recognition are collectively termed as sickness behaviours. Sickness behaviours make the organism inhospitable to an invading pathogen by impeding the pathogen's ability to proliferate (Hart, 1988). A cytokine-induced fever promotes immune cell activity and suppresses the pathogen proliferation (Dantzer, 2004).

There are two theorized pathways for cytokine-induced sickness behaviours: The neuronal and humoral routes. The neuronal pathway traverses directly through afferent sensory neurons that innervate the site of infection, such as vagal afferents of abdominal organs following an intraperitoneal injection. Research has shown that cytokines in the periphery (from an intraperitoneal injection of LPS) are recognized by microglia within the vagal nerve (Goehler et al., 1999). Activation of TLR and IL-expressing microglia within branches of the vagal nerve lead to the upregulation of factors such as cytokines, prostaglandins (PGE), and cyclo-oxygenase (Zhao et al., 2019). These factors interact with neurons in the vagal nerve and induce an inflammatory signal which is transmitted to the thalamic regions (Banks et al., 2015). Activation of these afferents lead to activation of lower brain regions implicated in sickness behaviours, such as the preoptic hypothalamus (Blomqvist & Engblom, 2018). Studies have shown that vagotomised mice that receive intraperitoneal (*ip*) injections of LPS display an attenuation in *c-fos* expression from secondary projections of the vagus nerve compared to non-vagotomised mice (Bluthe et al., 1991).

The other pathway of immune communication, the humoral route, acts indirectly through several converging mechanisms. Peripherally circulating pro-inflammatory molecules are sensed

by microglia in the circumventricular organs (CVOs), endothelial walls of brain blood vessels, and across the blood brain barrier (BBB; Rivest et al., 2000). The result is the production of cytokines and molecular intermediates by microglia and endothelial cells, such as interleukin factors, PGE, and NO. PGE and cytokines binds to neurons within the brainstem and hypothalamic areas (i.e. paraventricular nucleus [PVN] and MPOA) that are involved in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis and body temperature (Romanovsky, 2000).

Neuronal and humoral signalling converges into the HPA axis and surrounding thalamic areas. Within these areas, microglia (immune cells specific to the CNS) are normally in a quiescent phase, where they actively monitor neurotransmitter and neuron activity (Yang et al., 2010). Convergence of the inflammatory signals transition microglia into an "active" phase, which includes the initial production of cytokines, TNF-α, chemokines, and NO, followed by phagocytic functions (Yang et al., 2009). Cytokines (mainly IL-1) will circulate through CVOs to influence other brain structures. Intraperitoneal LPS injections enhance the expression of IL-1 (α and β) and TNF-α just 1 hour after injection. (Laye et al., 1994; Laye et al., 2000; Datson et al., 2004).

The upregulation of cytokines acts on neurons and astrocytes within the CNS environment. Research has shown that cytokines, such as Il-1β, act as excitatory molecules to neurons by increasing ceramide (intracellular lipid signaling molecule) synthesis, and downstream NMDA-mediated calcium influx (Viviani et al., 2003), resulting in an increased neuronal firing rate. Notably, high concentrations of cytokines can be debilitating to neuronal function. The direct binding of IL-1 to neurons induces the down-regulation of calcium channels, which is correlated

with inhibited long-term potentiation, altered ATP metabolism, and increased neural apoptosis (Schäfers & Sorkin, 2008).

Activation of afferent vagal pathways and increased cytokine-induced neural activation increases the firing rate of neurons in areas such as the hippocampus, amygdala, and hypothalamus (Ericsson et al., 1995). Activation of these areas during an immune challenge is associated with the initiation of sickness behaviours such as food aversion, fever, social withdrawal, pain sensitivity, and more. Notably, the exact molecular mechanism of neural activation that induces these behavioural changes remains elusive (Dantzer et al., 2008).

Along with inducing sickness behaviours, LPS-induced neuro-inflammation activates the HPA axis (Panagiotakopoulos & Neigh, 2014). Circulating cytokines (e.g. IL-1's) bind to the HPA and surrounding areas, and increase the firing rate of hypothalamic neurons (Ransohoff & Brown, 2012). Increased firing rates of hypothalamic neurons induces the release of corticotrophin-releasing hormone and arginine vasopressin into the hypophysial portal veins of the anterior pituitary. Adrenocorticotropic hormone (ACTH) is released from the anterior pituitary into general circulation. ACTH acts on the adrenal cortex to increase the synthesis and release of glucocorticoids from the adrenal glands into circulation (reviewed in Whitnall, 1993). Binding of glucocorticoids to target tissues induces a variety of physiological and behavioural responses such as increased heart rate, pupil dilation, increased blood pressure, and more (McCusker & Kelley, 2013).

The secretion of glucocorticoids is essential for the regulation of the inflammatory response**.** Glucocorticoids bind to receptors on immune cells, and induce intracellular signalling of glucocorticoid response elements (GREs), which alter NF- κβ and activator protein's pro-inflammatory transcriptional activity. During an acute immune challenge, GREs suppress

transcription of inflammatory genes, and thus decreasing the production of inflammatory molecules (reviewed in Sorrells & Sapolsky, 2007; Beishuizen & Lambertus, 2003). Alongside this, microglia clean infected areas of debris and toxic molecules, such as nitrate oxide.

At a certain point, high concentrations of glucocorticoids initiate a negative feedback mechanism. The feedback is initiated when glucocorticoids bind in high quantities to type I mineralocorticoid receptors (MRs), and type II glucocorticoid receptors (GRs; Datson, Morsink, Meijer, & de Kloet, 2008) within the hypothalamus and pituitary. Activation of these receptors inhibits the release of ACTH from the PVN, thus reducing glucocorticoid secretion. Overall, the suppressive action of glucocorticoids on immune cells prompts systems to return to a homeostatic state following immune activation.

The acute immune response differs across age and sex, due in part to circulating gonadal hormones and differential organization of interacting systems. Male and females have distinct immune responses after LPS exposure. Overall, estrogens act to enhance immune cell processes, whereas testosterone suppresses the functionality of immune cells (Cai et al., 2016; Walker, Besch-williford, & Keisler, 1994; Fox, 1977). In murine models of lupus, research with NZM2410 lupus-prone mice showed that ovariectomized females who were administered androgen had suppressed lupus development, while estrogen administration enhanced disease progression when compared to intact control female rats (Cunningham et al., 2016). Other research has shown that gonadectomised males display enhanced adrenal and immune responses to LPS, and administration of testosterone in these males reverses the effect, irrespective of age (Gaillard & Spinedi, 1998). As well, pubertal males display greater sickness behaviours (such as hypothermic responses to LPS) and take longer to recover from a sickness when compared to females (Cai et al., 2016). In addition to influencing the peripheral immune system, gonadal

hormones are directly correlated with microglial functionality in the central nervous system (CNS). Estrogen is positively correlated with microglial hippocampal expression in female rats (Pyter et al., 2013). Thus, estrogen is theorized to be protective against both peripheral and central inflammation after an acute immune challenge (Pyter et al., 2013).

Immune activation during puberty produces a differential response compared to adults. In pubertal mice, LPS exposure induces a hypo-responsive immune response compared to adults. At ten hours after LPS injection, pubertal male and female CD1 mice display decreased pro-inflammatory cytokines and increased anti-inflammatory cytokines in the blood compared to LPS-treated adults (Cai et al., 2016). This was also associated with reduced sickness behaviours (Sharma et al., 2019). It has been hypothesized that pubertal hypo-responsiveness is due to the neural re-organization occurring in HPA and HPG systems (Seckl & Meaney, 2004; Spencer et al., 2006).

Acute neuro-inflammation has beneficial outcomes for the host, and aids an organism in fighting an infection and returning to homeostasis without long-term damage. However, pre-occurring physical stressors can impact whether the acute inflammatory response is harmful or beneficial to an organism. This effect is more prominent in pubertal animals. Research has suggested that the gut microbiome is the key regulator in immune responsivity, and shifts in microbial composition can impact how immune cells respond to pathogenic exposure (Dinan & Cryan, 2017).

The microbiome

Microbial composition and function. The microbiome is a collection of bacteria that exists along the entire digestive tract, and contains 90% of the total microbes that colonize the

human body (Ilseung & Blaser, 2012). Species of bacteria contained within the digestive tract include the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Fusobacteria*, as well as non-bacterial species such as fungi, protists, archaea, and yeasts (Peterson et al., 2015). Within species (e.g. humans), there is high taxonomic diversity in microbe species due to environmental differences, such as diet, age, culture, and geography. While taxonomic diversity is high, the functional diversity of the microbiota is homologous across species (Lozupone et al., 2012). The bacteria within the microbiome exist in a balanced symbiotic role within the host, and play an essential role in physiological homeostasis. These benefits include immune system development and maintenance, vitamin and nutrient synthesis, intestinal permeability, and carbohydrate fermentation (See review: Engelborghs et al., 2004). The larger phyla of microbes (specifically *Bacteroidetes* and *Firmicutes*) are involved in fermentation of complex carbohydrates and fibres that cannot be broken down by the digestive system alone. The by-products of fermentation are secreted into the periphery and supplement distal systems throughout the body. This includes the production of: Short-chain fatty acids (SCFAs), amino acids, tryptophan, and oligopeptides (Zheng et al., 2011). SCFAs include molecules such as acetate, butyrate, and propionate (Macfarlane & Macfarlane, 2012).

The microbiome's fermentation processes indirectly regulate the production of certain neuro-hormones and derivatives that are essential for CNS signalling. It has been theorized that the microbiome is capable of synthesizing and regulating over sixty neuro-hormones and neurotransmitters. For example, *Lactobacillus* strains produce short-chain fatty acids (SCFAs), acetylcholine, histamine, and dopamine; *Bifidobacterium* strains produce serotonin, acetylcholine, SCFAs, and dopamine (Welcome, 2018).

Development of the microbiome. Initial colonization of the microbiome occurs during birth through ingestion of vaginal and skin bacteria of the mother (Dinan & Cryan, 2017). Delivery by C-section alters microbial composition to resemble the mother's skin more than vaginally delivered children. Animals born by C-section have delayed colonization by *Bacteroidetes* and *Bifidobacterium* (involved in milk oligosaccharide fermentation) as well as increased growth of enteric pathogen *C. difficile* (Borre et al., 2014).

The microbiome undergoes several compositional shifts throughout development. The shifts are to adjust for changes in food (e.g. introduction to solid food) and environment (daycares and school; Peterson et al., 2015). Recent studies have suggested that the maturation of the microbiome parallels critical periods of brain development, such as puberty. Pubertal individuals have increased microbial diversity compared to adults, with higher abundance of *Bifidobacterium* and *Clostridium* (Agans et al., 2011). As well, sexual differentiation of the brain during puberty is paired with sexual differentiation of the microbiome (Ober et al., 2008). Research in NOD/ShiLtJ mice has shown that the microbial composition of male mice deviates during puberty to acquire a unique composition in adulthood. Adult female microbial composition remains more similar to pre-pubescent male and female mice (Yurkovetskiy et al., 2013). Notably, castration of males prior to the pubertal period (4 weeks of age) maintained the pre-pubescent composition into adulthood, suggesting that circulating testosterone influences microbial composition in males.

The developmental trajectory of the gut microbiome during puberty moderates the impact of immuno-stressors in pubertal individuals, and can mitigate short-term and long-term changes in brain function and behaviour. In male and female CD-1 mice, supplementation with kefir (milk product containing live bacteria) during puberty prevents LPS-induced changes in the gut

microbiota, and reduces central cytokine expression after pubertal LPS exposure. In addition, probiotic supplementation mitigates enduring LPS-induced anxiety-like behaviours in adult males and depressive-like behaviours in adult female mice found in previous studies (Murray et al., 2019). The researchers concluded that a healthy microbiome is essential for mitigating immune stressors and reducing inflammatory responses in pubertal mice.

The gut-brain axis and development of the immune system

Development of peripheral autoimmunity. The gut microbiota exists in a bidirectional communication with the gut epithelial cells and the CNS. This communication is deemed the gut-brain axis, and each system is regulated through several physiological mechanisms. At the intestinal level, the microbiota interacts with the peripheral immune system through constant regulation to the gut environment. In a healthy gut, the microbiome and the peripheral immune system are physically separated by the intestinal barrier. This barrier is made up of layers of mucus, epithelial cells, anti-microbial markers (AMPs), and immune cells (Belkaid & Hand, 2014). Within the barrier, lymphocytes and epithelial cells monitor the production of bacterial metabolites to identify microbial composition and screen for pathogenic exposure (Lutgendorff & Akkermans, et al., 2008).

During development, initial bacterial exposure aids the immune system in differential recognition between commensal and pathogenic bacteria (Lazar et al., 2018). This differential recognition in neonates occurs through the binding of microbe-associated molecular patterns (MAMPs) to TLR2 receptors on immune and epithelial cells in the intestine walls (Belkaid & Hand, 2014). Continual exposure to commensal bacteria, such as *Bifidobacterium*, reduces TLR activation to specific MAMPs (Sekirov et al., 2010). As well, exposure to microbes such as

Lactobacilli increases immune tolerance to gram-positive bacteria through NFKB pathway suppression (Abt et al., 2012).

These early-life exposures to commensal bacteria induce differentiation and proliferation of immune cells implicated in intestinal immune homeostasis, such as neutrophils and T-cells (Lazar et al., 2018). Studies using germ-free mice have shown that adult germ-free mice have under-developed lymphoid structures, reduced immunoglobulin (IgA and IgG) producing cells, and immature T-cells (Kennedy et al., 2018). Supplementing germ-free mice with *B. fragilis* induces the proliferation of CD4+ T cells and white cells in the spleen (Mazmanian et al., 2005). Further research found that *lactobacilli spp.* supplementation in germ-free mice influenced the activation of natural killer (NK) cells, and shifted the balance of inflammatory cytokines within the intestinal mucosa (Christensen et al., 2002). The results suggested that the microbiome is essential for the full development and function of the immune system, and supplementation with a single component of the gut microbiome is sufficient to promote host immune cell maturation.

Homeostasis of the intestinal barrier is maintained by the microbiome and peripheral immune cells (Belkaid & Hand, 2014). Studies have reported that strains of *Lactobacillus* contribute to tight-junction protein repairs (Lutgendorff et al., 2008). Peripheral immune cells maintain the intestinal barrier by coating the intestinal walls with IgAs and AMPs, which prevent bacteria from breaching the lumen and inducing peripheral immune activation (Sekirov et al., 2010). AMPs—produced by lymphocytes—function to maintain gut microbiota proportions by suppressing cell division and inducing bacterial apoptosis. Notably, germ-free BALB/c mice display reduced tight-junction proteins (ZO-1 and occludin), and supplementation with a *Lactobacillus* probiotic mixture (*L. rhamnosus* and *L.Casei*) up-regulated the tight junction protein to resemble conventionally houses BALB/c mice (Kozakova et al., 2016).

The intestines are innervated by afferents of the vagal nerve that monitor the intestinal environment. The microbiome communicates with the brain through metabolite production. Dysbiosis-induced shifts in metabolites (such as an increase in inflammation) are detected by the lower structures of the brain (e.g. hypothalamus), and induce peripheral immune translocation and increased inflammation. Long-term disruptions in the intestinal environment has been correlated with depression and autoimmune disorders (McCusker & Kelley, 2013; Ruiz et al., 2005).

Development of neural autoimmunity. Peripheral immune signalling is detected by microglia in lower structures of the brain, such as the hypothalamus. Microglia are the resident immune cell of the brain, and are colonized in the brain prior to blood-brain barrier encapsulation during fetal development. In pre-pubertal development, microglia control rates of neurogenesis and apoptosis through the release of neuro-trophic and neuro-toxic factors (Cunningham et al., 2013). During puberty, microglia have been implicated in the synaptic remodeling of social and cognitive neural circuitry (Schafer et al., 2012; Paolicelli et al., 2011).

Microglia require microbial metabolites, such as SCFAs, for neuro-immune regulation (Lazar et al., 2018). Microbial-derived SCFAs are transported across the blood-brain barrier by mono-carboxylate transporters and absorbed by microglia and neurons, providing an essential energy source for cellular metabolism (Pellerin et al., 2005). In early development, SCFAs have been implicated in modulating intracellular calcium levels of neutrophils and subsequently influence cell signalling in the brain (Naccache et al., 1988; DeCastro et al., 2005). In germ-free models, the neural system is deprived of microbial metabolites, leaving the microglia and neural networks in an immature state (Abdel-Haq et al., 2019).

Germ-free mice have significant morphological differences in their microglia, compared to conventionally housed mice. This includes differences in cell volume, dendritic length, and branch points (Abdel-Haq et al., 2019). Alongside morphological differences, germ-free mice have reduced transcription rates for genes involved in autoimmune mechanisms, such as MyD88, which are highly expressed in microglia isolated from conventionally housed mice (Erny et al., 2015). In response to LPS, microglia from germ-free mice have impaired pro-inflammatory gene transcription (e.g. IL-1B, IL-6, and TNF), reduced activation of an amoeboid morphology, and increased neuro-degenerative markers (Souza et al., 2004; Erny et al., 2015). Colonization with a diverse microbial community facilitated the maturation of microglia to an adult-like phenotype, while colonization with select species (*Bacteroides distasonis*, *Lactobacillus salivarius*, and *Clostridium XIV*) maintained the germ-free phenotype (Erny et al., 2015). Notably, microglia isolated from adult mice treated with antibiotics display similar morphological and transcriptional profiles as microglia from germ-free mice (Erny et al., 2015; Thion et al., 2018). The results suggest that the continual presence of a diverse microbiome throughout lifespan is necessary for microglial maturation and homeostatic function.

While germ-free mice provide a broad picture of the importance of the microbiome in immune and neural development, these models are confounded by development and lack clinical relevance. Mice raised without a microbiome have significant immune system impairments, neural structural deficits, behavioural dysfunctions, and more (Kennedy et al., 2018). As well, germ-free mice lack information on sex differences, since microbial suppression often negates the sex differences found in conventionally housed mice (Elderman, de Vos, et al., 2018). As such, germ-free researchers cannot conclude the impact of the microbiome on neural autoimmunity, since the developmental trajectory of these systems are significantly impaired.

Research is transitioning to acute models of microbial dysbiosis, such as antibiotic treatments. Notably, models of acute dysbiosis in developing individuals are limited. The following review is based on antibiotic research conducted in adult male mice.

The gut-brain axis in acute microbial dysbiosis

Peripheral immune dysfunctions. Dysbiosis-induced changes in immune responsivity begins with a two-part shift in the gut ecosystem. In antibiotic-induced gut dysbiosis, antibiotic exposure suppresses dominant phyla, which allows for the proliferation of opportunistic bacteria (e.g. *C. difficile*; Kennedy et al., 2018). Reductions in dominant phyla results in the depletion of immuno-modulating molecules, as well as eliminating immuno-suppressive mechanisms (Lutgendorff et al., 2008). Intestinal immune cells detect the shifts in MAMPs in the lumen, and induce immune cell translocation to the gut.

In the lumen, immune cells up-regulate transcription of pro-inflammatory cytokines (Becattini et al., 2016), reactive oxidative species (ROS), and reactive nitrogen species (RNS; Yoon & Yoon, 2018). Opportunistic bacteria such as *Enterobacteriaceae* can utilize ROS and RNS species in their electron transport receptors for anaerobic respiration, further proliferating opportunistic bacteria growth. This maintains the acute inflammatory response in the intestinal space (Spees et al., 2013).

Increased inflammation places stress on the intestinal barrier. Alongside this, microbial dysbiosis disrupts barrier maintenance and epithelial cell function through deterioration of tight-junction proteins (Sekirov et al., 2010), promoting epithelial cell death and mucosal damage (Kelly et al., 2015). Over time, inflammatory stress creates gaps in the intestinal barrier, which leaks pro-inflammatory molecules, bacteria, and yeasts into peripheral circulation. Intestinal leakage is detected by vagal afferents and activates lower regions of the brain to induce a

peripheral immune response (Belkaid & Hand, 2014). Extended periods of microbial disruption lead to chronic activation of the peripheral immune system and HPA axis. Research has suggested that long-term activation induces dysfunctions in immune signalling and chronic low-grade inflammation. For example, a study by Ayres et al. (2012) showed that antibiotic-induced microbial suppression in C57BL/6 adult male mice significantly increased the likelihood of developing inflammatory disorders, such as colitis and sepsis, when compared to water-treated controls.

Central immune dysfunctions. Long-term disruptions in the gut environment has been shown to induce inflammatory stress in the central nervous system. There are three validated methods of communication between the microbiome and lower brain regions: (1) neural transmission through the vagal nerve, (2) humoral secretion through the blood, and (3) immune cell signalling through cytokine upregulation (see review: Gareau, 2014).

Research has shown that antibiotic-treated mice have higher central cytokine expression, increased central β-amyloid levels, and increased microglial activity compared to controls (Minter et al., 2016). Continual exposure to peripheral pro-inflammatory cytokines induces cellular dysfunctions in microglia (e.g. microtubule transport; Revett et al., 2013), which impairs microglial function. Impairments in microglial function (such as debris clearing) is associated with increased neuro-degeneration. *Drosophilia* infected with *Enterobacteria* display increased immune cell translocation to the brain, and increased markers associated with the TNF-JNK neuro-degenerative pathway (Wu et al., 2017).

Notably, supplementation with beneficial bacteria (e.g. probiotics) has the reverse effect on neuro-inflammation. For example, in human cell cultures, probiotic mixtures containing *Lactobacillus rhamnosus*, *Bifidobacterium lactis,* and *Bifidobacterium longum* reduce pro-

inflammatory cytokine expression (e.g. IL-8) by 70-80%, and increase the synthesis of anti-inflammatory cytokines (e.g. IL-10) within the culture (Sichetti et al., 2018). Further research has shown that *lactobacillus* probiotics decreases IL-8 expression through inhibition of the NF-κβ pathway (Frick et al., 2007). In germ-free Fischer rats, colonization with *Bifidobacterium* stimulates IL-6 production (anti-inflammatory cytokine) through TLR pathways (Ruiz et al., 2005).

The mechanism with which the gut microbiome influences neuro-inflammation remains elusive. One potential pathway for dysbiosis-induced neuro-inflammation is through neuro-modulating hormones and derivatives synthesized by the microbiome (Clarke et al., 2014). Studies have shown that vancomycin-treated male and female C57BL/6 mice display significant reductions in SCFA concentrations in colonic tissue, compared to controls (Gao et al., 2019). As well, similar studies have shown that treatments with antibiotic cocktails are associated with reduced mitochondrial gene expression in neurons and microglia, as well as increased neuronal cell death (Morgun et al., 2015). As such, decreases in energy sources (e.g. SCFAs) through gut dysbiosis can drastically increase host susceptibility to neuro-immune stressors, through mechanisms of cellular dysfunctions.

Further research has shown significant microglial disturbances in antibiotic mice models. One week of tamoxifen treatment in adult male and female mice significantly altered microglial transcriptional activity. In males, transcriptome shifts occurred in genes pertaining to stress and immune responsivity; Female transcriptome shifts occurred in genes regulating transcription and stress responsivity (Thion et al., 2018). A similar study by Erny et al. (2015) showed that antibiotic-treated mice have altered microglial cell morphology (e.g. increased dendritic spines)

and immature transcriptional phenotypes. Notably, administration of SCFAs to antibiotic-treated mice negated the deficits in microglial morphology.

Short-term microbial disruptions in adult mice has significant changes to acute immune responsivity. Notably, in adults, these physiological disruptions do not always result in long-term dysfunctions to the central nervous system (Erny et al., 2015). However, dysbiosis during development has been shown to have lasting changes in immune function, as well as the central nervous system (Desbonnet et al., 2015).

Acute microbial dysbiosis during development

Research on early life microbial dysbiosis has focused on the impact of dysbiosis on brain function and neuro-endocrine signalling. For example, antibiotic treatment from weaning to adulthood is associated with reduced anxiety, cognitive deficits, and reduced brain-derived neurotrophic factor expression (BDNF; Desbonnet et al., 2015; Bercik et al., 2011; Leclercq et al., 2017). Similar research on pubertal dysbiosis showed that administration of mixed antibiotics (ampicillin and cefoperazone) to 21 day old male BALB/c mice increased anxiety, decreased recognition memory, and increased depressive-like behaviours. This was associated with reduced hippocampal BDNF expression (Ceylani et al., 2018). Another study showed that two weeks of mixed antibiotic treatment (vancomycin, neomycin, metronidazole and ampicillin) in three-week old C57BL/6J mice reduced BDNF and tyrosine receptor kinase B expression in the hippocampus, but not the prefrontal cortex (Bistoletti et al., 2019).

In relation to neural auto-immunity, research has shown that low-dose penicillin in male and female BALB/c neonates altered blood-brain barrier permeability (increased TJ occludin expression), and increased baseline prefrontal IL-6 and IL-10 expression in both males and

females (Leclercq et al., 2017). Furthermore, antibiotic-induced gut dysbiosis in five-week old MS transgenic mice triggered spontaneous development of encephalomyelitis T cells and the induction of autoimmune encephalomyelitis in early adulthood (Yadav et al., 2017). In humans, a reduction in microbial diversity is associated with the onset of juvenile idiopathic arthritis (JIA) compared to healthy controls (De Filippo et al., 2019).

Current study

The literature assessing the influence of microbial dysbiosis on immune function is in its early phases, and the literature does not provide a clear picture of the mechanisms with which microbial dysbiosis impacts neuro-immune signalling in pubescent individuals. As well, the literature focuses on long-term microbial dysbiosis and its impact on behavioural and neuro-endocrine function, usually using adult male mice. As such, the literature lacks holistic and clinically relevant studies. Therefore, the objective of the current Master thesis is to determine the impact of acute pubertal microbial dysbiosis on acute immune responsivity in male and female mice, and to determine if this effect can be mitigated by probiotics. The hypothesis is that pubertal microbial dysbiosis and probiotic supplementation would alter immune responsivity in male and female mice. At five weeks of age, male and female CD-1 mice will be treated with mixed broad-spectrum antibiotics or water for seven days to induce microbial dysbiosis. During this period, mice will also be exposed to a probiotic (Lacidofil or Probio'Stick) or placebo in their drinking water for seven days. At six weeks of age (pubertal stress sensitive period), mice will be injected with LPS or saline. Sickness behaviours will be recorded for 8 hours after treatment. At 8 hours' post-injection, the mice will be euthanized and brain, blood samples, and intestinal weights will be collected. Brain tissues will be processed for hippocampal cytokine

expression using rt-qPCR. Blood samples will be examined for peripheral cytokine expression. Specific predictions are: (1) gut dysbiosis will potentiate LPS-induced sickness behaviours, plasma cytokine concentrations, and hippocampal cytokine mRNA expression, (2) probiotic supplementation will reduce LPS-induced sickness behaviours, plasma cytokine concentrations, and hippocampal cytokine mRNA expression, (3) probiotics will mitigate the effects of antibiotics on intestinal weights, LPS-induced sickness behaviours, plasma cytokine concentrations, and hippocampal cytokine mRNA expression, and (4) dysbiosis-induced inflammation will be sexually dimorphic, where males have higher LPS-induced sickness behaviours, plasma cytokine concentrations, and hippocampal cytokine mRNA expression. The following sections detail the methodologies discussed above.

2. Materials and methods

2.1. Animals

Two hundred and forty CD-1 male and female mice were shipped from Charles River Laboratories (Saint-Constant, Québec, Canada) in five cohorts of forty-eight mice at three weeks old. Mice were separated by sex and housed in groups of two on a reversed light cycle (lights off at 1000 h) under standard conditions (14 h:10 h light/dark cycle; 24 ± 2 °C; relative humidity of 40 ± 5). Mice were housed in polycarbonate Lexan housing cages (17 cm wide × 28 long × 12 cm high) that were bedded with Teklad Corn Cob bedding (Harlan Laboratories, Inc., Madison, WI, USA) and enriched with one square piece of Nestlet (Ancare Corp., Bellmore, NY, USA) and a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, Canada). The food (Harlan Laboratories, Inc., Madison, WI, US, T2018 – Global 18% rodent) and water were available *ad libitum*. All observational tests were completed during the dark phase under red

light unless specified. All experiments were approved by the Animal Care Committee of the University of Ottawa.

2.2. Antibiotic treatment

At five weeks of age, mice were administered 200μL mixed broad-spectrum antibiotic solution or distilled water through gavage twice daily for seven days. The antibiotic solution was made fresh daily and contained 15 mg/mL of ampicillin (No. BP1760-5, Fisher Scientific, Geel, Belgium), neomycin (No. 480125GM, EMD Millipore Corp, MA, USA), streptomycin (NO. BP910-50, Ala aesar, Fisher Scientific, Ottawa, ON), and 10 mg/mL of metronidazole (No. AC210340050, Acros Organics, New Jersey, USA) in distilled water. The treatments were administered at 0800 hours and 1800 hours, respectively. This dosage and treatment regimen have been shown to sufficiently suppress total microbial content (Zarrinpar et al., 2018).

2.3. Probiotic supplementation

At five weeks of age (simultaneous to the antibiotic treatments), mice were exposed to 1 billion CFU/mL of Lacidofil (*Lactobacillus rhamnosus* [95%] and *Lactobacillus helveticus* [5%]; Lallemand Inc., Montreal, QC, Canada), Probio'Stick (*Lactobacillus helveticus* [90%] and *Bifidobacterium longum* [10%]) or placebo (0% bacterial content) in their drinking water for seven days. Solutions were refreshed and weighed daily to assess consumption rates.

2.4. Body weight analyses

Body weights were measured at baseline (day before antibiotic and probiotic regiments) and daily throughout the treatment regimens. Changes in body weights were examined as a

percent change in body weight from baseline, where day zero (day before treatment regimen) was subtracted from day seven (last day of treatment) and converted to a percentage.

2.5. Lipopolysaccharide administration

Six-week old mice received an intraperitoneal (*ip*) injection of either 1.5 mg/kg of LPS (*Escherichia coli* seroptype O26:B6; L#3755; Sigma Chemical Co., St. Louis, MO, USA) or an equivalent volume of 0.9% sterile saline towards the end of the light cycle. This dose of LPS was chosen because it has been previously shown to induce sexually dimorphic sickness behaviours for approximately 24-48 hours (Cai et al., 2016).

2.6. Sickness monitoring

Sickness monitoring was conducted at 2, 4, 6, and 8 hours after injection. Assessment of the progression of sickness behaviours followed a non-invasive and unbiased approach with two raters blind to the experimental conditions (described in Kolmogorova et al., 2017). The raters visually assessed the mice for symptoms including lethargy (reduced locomotion), huddling (curled body posture), ptosis (drooping eyelids), and pilo-erection (erection of fur). At each time-point, the raters scored the total number of symptoms displayed by each mouse (one symptom = 1, two symptoms = 2, three symptoms = 3, four symptoms = 4). Sickness scores at each time-point were averaged from the two raters and used in statistical analyses.

2.7. Plasma extraction

At 8 hours after the saline or LPS treatment, mice were anesthetized with Euthanyl (Sodium pentobarbital; 500 mg/kg, *ip*). Mice were assessed for motor reflexes by gently

pinching their feet. Once no motor reflexes were detected, the mice were decapitated and trunk blood was collected into Microvette CB 300 K2E blood extraction tubes (Sarstedt AG & Co, Nümbrecht, Germany) that were coated with an anti-coagulant, EDTA. Tubes were kept at 4°C until plasma extraction. Within three hours of blood collection, samples were centrifuged at 1,000 x *g* at 20°C for 15 minutes to separate plasma. Plasma was extracted and stored in aliquots at -80°C.

2.8. Brain tissue extraction

Following decapitation, the brains were extracted and flash-frozen in liquid nitrogen and stored at -80°C until processing. The brain tissue was sliced with a cryostat at 600μM, and hippocampal tissue was extracted with 2.0mm Militex Biopsy Punchers into RNA-free tubes. Tubes were stored at -80°C until RNA extraction.

2.9. Whole intestine weight analyses

Following brain extraction, the whole intestine was cut at the distal colon and the duodenum and weighed. Whole intestine weights were recorded across groups and were used as a validated method to assess the efficacy of antibiotic treatments on intestinal physiology and microbial content (Zarrinpar et al., 2018).

2.10. Real-time quantitative PCR

mRNA was extracted from hippocampal tissue using QIAzol Lysis Reagent (No. 79306; Thermo-Fisher Scientific) and precipitated with Glycogen (No. 10814-010; Invitrogen) and isopropanol. Extracted RNA was then incubated with gDNA wipeout buffer to remove genomic

DNA prior to cDNA synthesis. cDNA was synthesized with the QuantiTect Reverse Transcription kit (No. 205311; QIAGEN). The products of the cDNA synthesis step were used in subsequent real-time quantitative PCR. Relative gene expression was assessed in 10μL reactions using the CFX96 Touch Real-Time PCR Detection System. All primers were ordered from Integrated DNA technologies. Primer efficiency was determined using the slope between RNA quantity and cycle thresholds with Bio-rad software. All primer pairs achieved reaction efficiency between 90-110%. Dilutions factors for primers was 0.3μM for real-time PCR. The primer sequences are as follows: β-actin forward: GAACCCTAAGGCCAACCGTG, reverse: GGTACGACCAGAGGCATACAGG; IL-1β forward: TCTTGGGACTGATGCTGGTG, reverse: CAGAATTGCCATTGCACAACTC; TNFα forward: GCCTATGTCTCAGCCTCTTCTC, reverse: GCCATTTGGGAACTTCTCATCC; IL-6 forward: GCCTTCTTGGGACTGATGCT, reverse: GCCATTGCACAACTCTTTTCTC. B-actin was used as a housekeeping gene for all samples, and did not significantly change across experimental conditions. For each reaction, the quantitative threshold amplification cycle number (CQ) was determined, and the $2-\Delta\Delta Cq$ method was used to calculate the relative gene expression of each gene.

2.11. Multiplex immunoassay

Plasma concentrations of IL-1β, IL-6, IL-10, IL-12 (*p70*), IFN-γ, and TNF-α were measured with a multiplex Luminex immunoassay. Multiplex kits (No. MCYTOMAG-70K-05; Millipore-Sigma) were used according to the supplier's instructions, and plasma samples were plated in duplicates. Each plate contained one pooled sample as a reference across plates. The

MAGPIX system was used to measure the final cytokine concentrations. Samples with %CVs greater than 10% were excluded from the analyses.

2.12. Experimental procedures

At 5 weeks of age, the mice received twice daily either mixed broad-spectrum antibiotics or distilled water through gavage for seven days. During this period, mice were exposed to either Lacidofil, Probio'Stick or placebo in their drinking water for seven days. At six weeks of age (pubertal stress sensitive period), mice were injected with either saline or LPS. Sickness behaviours were recorded for 8 hours following treatment. At 8 hours, the mice were euthanized, and brain, blood, and intestinal weights were collected. Brain tissues were processed for central cytokine expression using RT-qPCR. Blood samples were examined for peripheral cytokine expression using Multiplex (Figure 1).

2.13. Statistical analyses

All statistical analyses were performed using IBM SPSS v20 software. Cases that exceeded the 1.5 interquartile range in z-score analyses (probiotic consumption, body weight and sickness behaviour data) and boxplot analyses (intestine weights, rt-qPCR, and multiplex data) were considered statistical outliers and were winsorized to the next outer-most score within the 1.5 interquartile range (Hastings et al., 1947). For all measures, 2 x 2 x 3 x 2 ANOVAs were performed for sex (male or female), antibiotic treatment (AMNS or water), probiotic (lacidofil, probio'stick, or placebo), and LPS treatment (LPS or saline). For measures of sickness behaviours and probiotic consumption data (repeated measures), Greenhouse-Geisser corrections was applied to *F*-values that violated Mauchly's test of sphericity. Statistically significant effects

were followed by pairwise comparisons with Bonferroni corrections, when appropriate. Measures of effect sizes were estimated using partial eta-squared (η_{p2}). Statistical significance was set to $p < .05$.

3. Results

3.1. Consumption rates of probiotic supplements.

The 2 x 2 x 3 x 2 mixed ANOVA violated Mauchly's Test of Sphericity ($p < 0.05$), and all within-subject effects were assessed with Greenhouse-Geisser corrections. The ANOVA revealed significant within-subjects main effect of time ($F_{(4.4,1000)} = 42.56$, $p < 0.05$, $\eta_{p2} = .16$), and significant time x sex ($F_{(4.4,1000)} = 6.38$, $p < 0.05$, $\eta_{p2} = .03$), time x antibiotics ($F_{(4.4,1000)} = 2.36$, $p < 0.05$, $\eta_{p2} = .01$), time x probiotics ($F_{(8.85,1000)} = 2.73$, $p < 0.05$, $\eta_{p2} = .02$), and time x sex x antibiotic x probiotic ($F_{(8.85,1000)} = 1.88$, $p = 0.05$, $\eta_{p2} = .02$) interactions. The ANOVA also revealed significant between-subjects main effects of sex ($F_{(1,226)} = 5.053$, $p < 0.05$, $\eta_{p2} = .02$), antibiotics ($F_{(1,226)} = 26.12$, $p < 0.05$, $\eta_{p2} = .10$), and probiotics ($F_{(2,226)} = 4.42$, $p < 0.05$, $\eta_{p2} = .04$).

Pairwise comparisons showed that regardless of antibiotic and probiotic treatments, males drank more than females ($MD = 0.42$, $SE = .19$), specifically on days 3, 4, and 5 ($MD = 0.76$, $SE = .24$, $p < 0.05$; $MD = 0.49$, $SE = .22$, $p < 0.05$; $MD = 1.41$, $SE = .34$, $p < 0.05$, respectively). Regardless of sex and probiotic treatment, antibiotic-treated mice drank less than water-treated mice ($MD = -.95$, $SE = .19$, $p < 0.05$), on days 1, 2, 3, 4, and 6 ($MD = -1.63$, $SE = .23$, $p < 0.05$; $MD = -0.91$, $SE = .25$, $p < 0.05$; $MD = -1.09$, $SE = .24$, $p < 0.05$, $MD = -.76$, $SE = .22$, $p < 0.05$, $MD = -.98$, $SE = .28$, $p < 0.05$, respectively). Placebo-treated mice drank significantly more than probio'stick-treated mice ($MD = 0.67$, $SE = .23$, $p < 0.05$), specifically on

days 2, 3, and 5 (*MD* = 0.75, *SE* = .31, $p < 0.05$; *MD* = 0.73, *SE* = .29, $p < 0.05$; *MD* = 1.24, *SE* = .41, $p < 0.05$, Figure 2). Finally, pairwise comparisons showed that antibiotic-treated males exposed to placebo consumed significantly more than counterparts exposed to lacidofil on days 1, 2, 3, and 5 (*MD* = 1.35, *SE* = .55, $p < 0.05$; *MD* = 1.80, *SE* = .62, $p < 0.05$; *MD* = 1.75, *SE* = .58, $p < 0.05$; *MD* = 1.95, *SE* = .82, $p = 0.05$, respectively; Figure 2a). As well, antibiotic-treated males exposed to placebo consumed significantly more than counterparts exposed to probio'stick on days 5 and 6 (*MD* = 2.35, *SE* = .82, $p < 0.05$; *MD* = 1.70, *SE* = .68, $p < 0.05$, respectively; Figure 2a). Antibiotic-treated females exposed to probio'stick consumed significantly less than counterparts exposed to placebo or lacidofil groups on day 7 (*MD* = -2.65, *SE* = .88, $p < 0.05$; *MD* = -2.56, *SE* = .90, $p < 0.05$, respectively; Figure 2b). Water-treated females exposed to lacidofil consumed significantly more than counterparts exposed to placebo and probio'stick on day 6 (*MD* = 1.75, *SE* = .68, $p < 0.05$; *MD* = 1.95, *SE* = .68, $p < 0.05$, respectively; Figure 2b).

3.2. Body weight changes.

The 2 x 2 x 3 x 2 ANOVA revealed a significant main effect of sex ($F_{(1,228)} = 14.82$, $p < 0.05$, $\eta_{p2} = .06$), where males had significantly more percent body weight change after seven days of treatment than females (*MD* = 2.11, *SE* = .55). There was also a significant sex x antibiotics interaction ($F_{(1,228)} = 6.03$, $p < 0.05$, $\eta_{p2} = .03$). Pair-wise comparisons showed that antibiotic-treated males gained significantly less weight compared to water-treated counterparts (*MD* = -2.31, *SE* = 0.78, $p < 0.05$; Figure 3a). There was no significant body weight change in the females (Figure 3b).

3.3. Sickness behaviours.

The 2 x 2 x 3 x 2 mixed ANOVA violated Mauchly's Test of Sphericity ($p < 0.05$), and all within-subject effects were assessed with Greenhouse-Geisser corrections. The ANOVA produced significant within-subjects main effect of time ($F_{(2.7,589)} = 581.58$, $p < 0.01$, $\eta_{p2} = .73$) and significant time x sex ($F_{(2.7,589)} = 2.74$, $p < 0.05$, $\eta_{p2} = .01$), time x LPS treatment ($F_{(2.7,589)} = 600.33$, $p < 0.01$, $\eta_{p2} = .74$), and time x sex x LPS treatment ($F_{(2.7,589)} = 4.44$, $p < 0.05$, $\eta_{p2} = .02$) interactions. The ANOVA also revealed significant main effects of sex ($F_{(1,214)} = 11.03$, $\eta_{p2} = .05$, $p < 0.01$) and LPS treatment ($F_{(1,214)} = 2671.863$, $\eta_{p2} = .93$, $p < 0.001$), and significant sex x LPS treatment ($F_{(1,214)} = 4.90$, $p < 0.05$, $\eta_{p2} = .03$), sex x antibiotics ($F_{(1,214)} = 7.53$, $p < 0.05$, $\eta_{p2} = .03$), LPS treatment x antibiotics ($F_{(1,214)} = 16.34$, $p < 0.01$, $\eta_{p2} = .07$), antibiotic x probiotic ($F_{(2,214)} = 2.98$, $p = 0.05$, $\eta_{p2} = .03$), and sex x LPS treatment x antibiotics ($F_{(1,214)} = 8.90$, $p < 0.05$, $\eta_{p2} = .04$) interactions.

Pairwise comparisons of the time x sex x LPS treatment interactions showed that saline-treated males had significantly more sickness scores than saline-treated females (*MD* = .24, *SE* = .06, $p < 0.05$) at 2, 4, 6, and 8 hours (*MD* = .21, *SE* = .09, $p < 0.05$; *MD* = .25, *SE* = .08, $p < 0.05$; *MD* = .25, *SE* = .08, $p < 0.05$; *MD* = .25, *SE* = .08, $p < 0.05$). LPS-treated males displayed significantly more sickness than saline-treated females at 2 hours (*MD* = .31, *SE* = .09, $p < 0.001$). Interpretations of the sex x antibiotic interaction showed that antibiotic-treated males had significantly more sickness behaviours compared to their female counterparts (*MD* = .26, *SE* = .06, $p < 0.01$). Interpretations of the antibiotic x probiotic interaction showed that antibiotic-treated mice exposed to lacidofil had significantly less sickness scores compared to antibiotic-treated counterparts exposed to placebo-treated or to probio'stick (*MD* = -.21, *SE* = .08, $p < 0.05$; *MD* = -.23, *SE* = .08, $p < 0.05$, respectively). Interpretation of the three-way interaction of sex x LPS treatment x antibiotics showed that saline-treated antibiotic-treated males displayed

significantly more sickness behaviours compared to saline-treated antibiotic-treated female counterparts (*MD* = .49, *SE* = .09, $p < 0.01$). Pair-wise comparisons also showed that LPS-treated antibiotic-treated males and females displayed significantly less sickness behaviours at 8HRs after treatment compared to their LPS-treated water-treated counterparts (*MD* = -.28, *SE* =.12, $p < 0.05$; *MD* = -.23, *SE* = .12, $p = 0.06$, respectively; Figure 4).

3.4. Whole intestinal weights.

The 2 x 2 x 3 x 2 ANOVA found significant main effects of sex ($F_{(1,212)} = 180.62$, $p < 0.01$, $\eta_{p2} = .46$) LPS treatment ($F_{(1,212)} = 372.77$, $p < 0.01$, $\eta_{p2} = .64$), and antibiotics ($F_{(1,212)} = 515.78$, $p < 0.01$, $\eta_{p2} = .71$). As well, significant sex x LPS treatment ($F_{(1,212)} = 7.949$, $p < 0.01$, $\eta_{p2} = .04$), LPS treatment x antibiotics ($F_{(1,212)} = 25.23$, $p < 0.01$, $\eta_{p2} = .11$), and treatment x probiotics ($F_{(1,212)} = 3.93$, $p < 0.05$, $\eta_{p2} = .04$) interactions were found.

Pairwise comparisons showed males had significantly lower intestinal weight after LPS treatment compared to LPS-treated females (*MD* = .40, *SE* = .06, $p < 0.01$). As well, pairwise comparisons of the LPS treatment x antibiotic interaction showed that antibiotic-treated mice had significantly lower intestinal weight after LPS, when compared to water-treated counterparts (*MD* = -.94, *SE* =.06, $p < 0.01$). Finally, the pairwise comparisons of treatment x probiotics showed that mice who received lacidofil had significantly higher intestinal weight after LPS compared to placebo-treated LPS-treated counterparts (*MD* = .20, *SE* = .08, $p < 0.01$, Figure 5).

3.5. Assessment of hippocampal mRNA cytokine expression with RT-qPCR.

3.5.1. IL1β mRNA expression.

The ANOVA found a significant main effects of LPS treatment ($F_{(1,93)} = 54.93$, $p < 0.01$, $\eta_{p2} = .37$). Pairwise comparisons showed that regardless of sex, antibiotic, and probiotic treatments, LPS-treated mice had significantly greater IL1β mRNA expression compared to saline-treated counterparts (*MD* = 46.29, *SE* = 6.25, $p < 0.01$; Figure 6A, 7A).

3.5.2. TNFα mRNA expression.

The ANOVA revealed a significant main effect of LPS treatment ($F_{(1,93)} = 45.76$, $p < 0.001$, $\eta_{p2} = .33$), as well as significant sex x antibiotics ($F_{(1,93)} = 6.14$, $p < 0.05$, $\eta_{p2} = .06$) and sex x LPS treatment ($F_{(1,93)} = 5.36$, $p < 0.05$, $\eta_{p2} = .06$) interactions. There was also a trend towards a significant sex x LPS treatment x antibiotics interaction ($F_{(1,93)} = 3.73$, $p = 0.06$, $\eta_{p2} = .04$). Pairwise comparisons showed that regardless of antibiotic and probiotic treatments, LPS-treated mice had significantly greater TNFα mRNA expression compared to saline-treated counterparts (*MD* = 4.87, *SE* = .72, $p < 0.01$); LPS-treated females had significantly greater TNFα mRNA expression compared to LPS-treated male counterparts (*MD* = 2.57, *SE* = 1.00, $p < 0.05$). As well, regardless of LPS or probiotic treatment, antibiotic-treated males displayed significantly greater TNFα mRNA expression compared to water-treated males (*MD* = 2.10, *SE* = 1.01, $p < 0.05$). Pairwise comparisons of the trending three-way interaction showed that regardless of probiotic treatment, antibiotic-treated males and females exposed to LPS displayed significantly greater TNFα mRNA expression compared to their water-treated counterparts (*MD* = 3.64, *SE* = 1.42, $p < 0.05$; *MD* = 2.71, *SE* = 1.42, $p = 0.06$, respectively; Figure 6B).

3.5.3. IL-6 mRNA expression.

The ANOVA found significant main effects of sex ($F_{(1, 93)} = 73.93$, $p < 0.05$, $\eta_{p2} = .44$) and LPS treatment ($F_{(1,93)} = 139.72$, $p < 0.01$, $\eta_{p2} = .60$. There were also significant sex x LPS treatment ($F_{(1,93)} = 52.44$, $p < 0.01$, $\eta_{p2} = .36$), sex x probiotic ($F_{(2,93)} = 3.97$, $p < 0.05$, $\eta_{p2} = .08$),

and sex x LPS treatment x probiotic ($F_{(2,92)}$ = 4.48, $p < 0.05$, η_{p2} = .09) interactions. Pairwise comparisons showed that regardless of antibiotic and probiotic treatments, LPS-treated mice displayed greater IL-6 mRNA expression compared to saline treated counterparts (*MD* = 56.50, *SE* = 4.78, $p < 0.01$); LPS-treated males had significantly greater IL-6 mRNA expression compared to LPS-treated female counterparts (*MD* = 75.72, *SE* = 6.66, $p < 0.01$). As well, regardless of antibiotic treatment, LPS-treated males supplemented with placebo had significantly greater IL-6 mRNA expression compared to lacidofil (*MD* = 56.10, *SE* = 11.53, $p < 0.05$) and probio'stick treated males (*MD* = 27.04, *SE* = 11.53, $p = 0.06$, respectively; Figure 7C).

3.6. Assessment of peripheral inflammation with multiplex immunoassays.

3.6.1. IFNγ plasma concentrations.

The ANOVA found a significant main effect of LPS treatment ($F_{(1,89)}$ = 188.05, $p < 0.01$, η_{p2} = .68). There was were also significant antibiotic x probiotic ($F_{(2,93)}$ = 6.07, $p < 0.01$, η_{p2} = .12) and LPS treatment x antibiotics x probiotic ($F_{(2,93)}$ = 6.014, $p < 0.01$, η_{p2} = .12) interactions. Pairwise comparisons showed that regardless of sex, antibiotic, and probiotic treatments, LPS-treated mice displayed significantly greater IFNγ concentration compared to saline-treated counterparts (*MD* = 1522.31, *SE* = 111.01, $p < 0.01$). As well, regardless of sex and LPS treatment, pairwise comparisons of the antibiotic x probiotic interaction showed that antibiotic-treated mice supplemented with lacidofil had a significantly lower IFNγ concentration than probio'stick-treated counterparts (*MD* = -616.03, *SE* = 190.55, $p < 0.01$). Further analysis showed that the antibiotic-treated mice supplemented with lacidofil had significantly less IFNγ expression compared to their water-treated counterparts (*MD* = -540.63, *SE* =198.78, $p < 0.01$);

while antibiotic-treated mice supplemented with probio'stick had significantly greater IFNγ expression compared to their water-treated counterparts (*MD* = 406.02, *SE* =193.33, $p < 0.05$).

Pairwise comparisons of the LPS treatment x antibiotic x probiotic interaction showed that in the water-treated groups, LPS-treated mice that were supplemented with probio'stick had significantly lower IFNγ concentration compared to their placebo-treated counterparts (*MD* = -754.66, *SE* = 261.44, $p < 0.05$). As well, there was a trend towards lower IFNγ concentration in the probio'stick group compared to their lacidofil treated counterparts (*MD* = -654.79, *SE* = 277.29, $p = 0.06$). In the antibiotic-treated groups, LPS-treated mice had significantly less IFNγ concentration when supplemented with lacidofil than counterparts supplemented with probio'stick (*MD* = -1233.25, *SE* = 269.48, $p < 0.01$), and counterparts that only received the placebo (*MD* = -746.88, *SE* = 251.93, $p < 0.05$). As well, LPS-treated mice exposed to water and lacidofil had significantly greater IFNγ concentration than antibiotic-treated counterparts (*MD* = 1074.03, *SE* = 277.29, $p < 0.01$), while LPS-treated mice exposed to water and probio'stick had significantly less IFNγ concentration compared to antibiotic-treated counterparts (*MD* = -814.00, *SE* = 269.48, $p < 0.01$; Figure 8).

3.6.2. IL1β plasma concentrations.

The ANOVA found a significant main effect of LPS treatment ($F_{(1,89)} = 89.61$, $p < 0.01$, $\eta_{p2} = .50$). There was also a trend towards a main effect of probiotics ($F_{(1,89)} = 2.93$, $p = 0.06$, $\eta_{p2} = .06$) and an LPS treatment x probiotic interaction ($F_{(1,89)} = 2.94$, $p = 0.06$, $\eta_{p2} = .06$). Pairwise comparisons showed that regardless of sex, antibiotic, and probiotic treatments, LPS-treated mice had significantly greater IL1β concentration compared to saline-treated mice (*MD* = 16.31, *SE* = 1.72, $p < 0.01$). Pairwise comparisons of the LPS treatment x probiotic interaction showed that the LPS-treated mice supplemented with placebo had significantly greater IL1β

concentration compared to lacidofil treated (*MD* = 9.20, *SE* = 2.91, $p < 0.01$), and probio'stick treated counterparts (*MD* = 8.07, *SE* = 2.86, $p < 0.05$; Figure 9).

3.6.3. IL-6 plasma concentrations.

The ANOVA found significant main effects of LPS treatment ($F_{(1,89)} = 85.21$, $p < 0.01$, $\eta_{p2} = .49$) and sex ($F_{(1,89)} = 5.47$, $p < 0.05$, $\eta_{p2} = .06$). There was also a significant LPS treatment x sex ($F_{(1,89)} = 5.47$, $p < 0.05$, $\eta_{p2} = .06$) interaction. Pairwise comparisons showed that regardless of sex, antibiotic, or probiotic treatment, LPS-treated mice displayed significantly greater IL-6 concentration compared to saline-treated mice (*MD* = 1404.15, *SE* = 151.11, $p < 0.01$). As well, regardless of LPS treatment, antibiotic, and probiotic treatments, male mice had significantly greater IL-6 expression compared to their female counterparts (*MD* = 355.73, *SE* = 152.11, $p < 0.05$). Investigation of the LPS treatment x sex interaction showed that LPS-treated males had significantly greater IL-6 concentration compared to their female counterparts (*MD* = 711.49, *SE* = 210.78, $p < 0.01$; Figure 10).

3.6.4. IL-10 plasma concentrations.

The ANOVA found a significant main effect of LPS treatment ($F_{(1,89)} = 152.86$, $p < 0.01$, $\eta_{p2} = .63$). Pairwise comparisons showed that regardless of sex, antibiotic, and probiotic treatment, LPS-treated mice had significantly higher IL-10 concentration compared to saline-treated mice (*MD* = 158.78, *SE* = 12.84, $p < 0.01$; Figure 11).

3.6.5. IL-12 plasma concentrations.

The ANOVA found a significant main effect of LPS treatment ($F_{(1,89)} = 90.57$, $p < 0.01$, $\eta_{p2} = .50$). As well, there was a trend towards a LPS treatment x antibiotic x probiotic ($F_{(2,89)} = 2.97$, $p = 0.06$, $\eta_{p2} = .06$) interaction. Pairwise comparisons showed that regardless of sex, antibiotic, and probiotic treatments, LPS-treated mice had significantly greater IL-12 expression

compared to saline-treated counterparts (*MD* = 12.89, *SE* = 1.36, $p < 0.01$). Pairwise comparisons of the LPS treatment x antibiotic x probiotic interaction showed that LPS- and antibiotic-treated mice exposed to placebo had significantly greater IL-12 concentration compared to the water-treated counterparts (*MD* = 9.36, *SE* = 3.08, $p < 0.01$). As well, for antibiotic-treated mice exposed to LPS, supplementation with lacidofil significantly lowered IL-12 expression compared to placebo-treated counterparts (*MD* = 11.47, *SE* = 3.08, $p < 0.01$; Figure 12).

3.6.6. TNFα plasma concentrations.

The ANOVA found a significant main effect of LPS treatment ($F_{(1,108)} = 162.40$, $p < 0.01$, $\eta_{p2} = .65$). Pairwise comparisons showed that regardless of sex, antibiotic, and probiotic treatments LPS-treated mice displayed significantly greater TNFα concentration compared to saline-treated counterparts *(MD* = 29.83, *SE* = 2.34, $p < 0.01$; Figure 13).

4. Discussion.

The gut microbiome plays a central role in the development and maintenance of the immune system (Leclercq et al., 2017; Murray et al., 2020; Souza et al., 2004; Desbonnet et al., 2015). Several studies have shown that dysfunctions of the microbiome in adults leads to short-term and long-term changes in immune function (Erny et al., 2015; Elderman et al., 2018; Lazar et al., 2018; Murray et al., 2019). However, research assessing the effect of pubertal gut dysbiosis on acute immune responsivity in male and female mice was lacking. Thus, the objective of this thesis was to investigate the impact of pubertal gut microbial changes on acute immune responsivity in male and female mice, and to determine if this effect can be mitigated by probiotics. We hypothesized that (1) gut dysbiosis would potentiate LPS-induced sickness

behaviours, plasma cytokine concentrations, and cytokine mRNA expression in the hippocampus, (2) probiotic supplementation would reduce LPS-induced sickness behaviours, plasma cytokine concentrations, and cytokine mRNA expression in the hippocampus, (3) probiotics would mitigate the effects of antibiotics on intestinal weights, LPS-induced sickness behaviours, plasma cytokine concentrations, and cytokine mRNA expression in the hippocampus, and (4) dysbiosis-induced inflammation would be sexually dimorphic, where males would have higher LPS-induced sickness behaviours, plasma cytokine concentrations, and cytokine mRNA expression in the hippocampus. First, the results found that the antibiotic treatment in pubertal mice induced changes in acute immune responsivity. Post-euthanasia assessments of the intestinal environment showed that the antibiotic treatment significantly increased whole intestinal weights; LPS treatment in antibiotic-treated mice significantly decreased intestine weights compared to their water-treated counterparts. Assessments of inflammatory markers showed that antibiotic-treated males exposed to saline displayed significantly more hippocampal TNFα mRNA expression compared to their water-treated counterparts. The antibiotic treatment also significantly increased LPS-induced IL-12 plasma concentrations, as well as LPS-induced hippocampal TNFα mRNA expression. Observational analyses showed that the antibiotic treatment significantly reduced LPS-induced sickness behaviours at eight hours' post-injection. Secondly, pubertal probiotic supplementation altered acute immune responsivity. Post-euthanasia assessments of the intestinal environment showed that lacidofil-supplemented mice had significantly higher LPS-treated intestinal weight compared to placebo-treated counterparts. Assessments of inflammatory markers showed that probio'stick supplementation significantly reduced LPS-induced IL1β and IFNγ plasma concentrations; lacidofil supplementation significantly reduced LPS-induced IL1β plasma

concentrations. As well, lacidofil and probio'stick supplementation reduced hippocampal LPS-induced IL-6 mRNA expression in male mice. Lastly, probiotic supplementation mitigated specific antibiotic-induced changes in acute immune responsivity. Antibiotic-treated mice supplemented with lacidofil had significantly lower LPS-induced IFNγ and IL-12 plasma concentrations compared to placebo-treated mice. Antibiotic-treated mice supplemented with lacidofil displayed significantly less sickness behaviours compared to placebo and probio'stick treated counterparts. Overall, the results suggested that changes in microbial composition during the pubertal period influence acute immune responsivity in male and female mice.

4.1. Impact of antibiotic treatment on acute immune responsivity

The current thesis showed that antibiotic-induced microbial dysbiosis was associated with significant disruptions in the intestinal environment, LPS-induced inflammation, and observed sickness behaviours. First, antibiotic-treated mice had significantly higher whole intestinal weights compared to water-treated mice. Intestinal weight changes have been used to confirm the broad effects of antibiotics on the intestinal environment (Zarrinpar et al., 2018; Reikvam et al., 2011). Increased intestinal weight is associated with reduced bacterial content, impaired digestive function, water retention, and increased intestinal inflammation (Reikvam et al., 2011). As well, the results showed that antibiotic-treated mice exposed to LPS had significantly lower intestinal weights compared to their LPS- and water-treated counterparts. An intraperitoneal injection of LPS induces intestinal fluid excretion, diarrhea, and increases gut permeability (Williams et al., 2013), thus reducing intestine weights. Our study showed that antibiotic-treated mice exposed to LPS had an exaggerated LPS-induced weight loss compared to their water-treated counterparts. This additional intestine weight loss may be due to increased intestinal

permeability. Previous research has shown that ampicillin treatments in C5BL/6 mice reduce tight-junction proteins, as well as increase intestinal permeability and endotoxin concentrations in the intestinal lumen (Ying Shi et al., 2018). Therefore, increased permeability of the intestinal barrier in our mice may have increased LPS concentrations in the lumen, and exaggerated LPS-induced water excretion and intestinal weight loss (Benoit et al., 1998; Ge et al., 2000).

Antibiotic-induced disruptions of the intestinal environment was also associated with changes in peripheral inflammation. Our results showed that mice exposed to antibiotics had significantly greater LPS-induced IL-12 plasma concentrations compared to water-treated mice. This result is consistent with our first hypothesis, as well as with previous research. Studies have consistently shown that antibiotic treatments increase peripheral inflammation (Becattini et al., 2016; Yoon & Yoon, 2018; Spees et al., 2013; Malys et al., 2015). The cytokine IL-12 is a pro-inflammatory cytokine produced during bacterial exposure (Frick et al., 2007; Trinchieri, 2003), and functions as a pro-inflammatory moderator by activating and regulating immune cells (Wojno et al., 2019). The increase of LPS-induced IL-12 concentrations in antibiotic-treated mice is likely due to disruptions of the intestinal barrier. Previous research has shown that antibiotic treatments increase the translocation of microbes and bacterial endotoxins across the epithelial barrier into peripheral circulation (Paulos et al., 2007; Round et al., 2010). Therefore, increased bacterial concentrations prior to an LPS exposure would lead to increased activation of TLR-expressing immune cells, and greater IL-12 concentrations during an acute immune challenge (Brandl et al., 2008).

Antibiotic-induced disruptions of the intestinal environment and aggravation of the peripheral immune response was also associated with changes in central inflammation. The results showed that regardless of LPS treatment, antibiotic-treated males displayed significantly

greater hippocampal TNFα mRNA expression compared to water-treated counterparts. After LPS exposure, antibiotic-treated male and female mice displayed significantly greater hippocampal TNFα mRNA expression than water-treated counterparts. The results were consistent with our second and fourth hypotheses, as well as with previous research (Leclercq et al., 2017; Lazar et al., 2018; Minter et al., 2016; Wu & Wu, 2012). The cytokine TNFα is involved in pro-inflammatory processes and in cellular degeneration (van Kralingen et al., 2013). The increased expression of neuro-degenerative markers in the hippocampus may have stemmed from antibiotic-induced disruptions of the intestinal environment. Previous research has shown that microbial dysbiosis is associated with increased intestinal inflammation and cytokine-induced activation of the vagal nerve (Forsythe et al., 2014). Chronic vagal activation would lead to an increased firing rate of cholinergic neurons projecting from basal ganglia and nuclei, which lead to thalamic and primary sensorimotor regions (Wang & Wang, 2016). Continuous neural excitation of these regions is associated with stress-induced alterations of NMDA-mediated calcium influx (Viviani et al., 2003; Schäfers & Sorkin, 2008), pro-inflammatory cytokine production (Gao et al., 2019), oxidative marker expression (Morgun et al., 2015; Abdel-Haq et al., 2019), and apoptosis (Thion et al., 2018). Therefore, increased degenerative markers in antibiotic-treated mice may be due to chronic hyperactivity of hippocampal cells. These disruptions in neural tissue would be particularly impactful during a critical period of development, and would sensitize neural pathways to degeneration and cellular apoptosis (Revett et al., 2013; Block & Hong, 2005; Bilbo, 2010; Bilbo et al., 2005).

As well, the influence of circulating hormones on immune cells would explain the sexually dimorphic effect of antibiotics on hippocampal TNFα mRNA expression in male mice. Previous research has shown that females have protective mechanisms in acute immune

challenges (Tanapat et al., 1999; Pyter et al., 2013). Estradiol and progesterone act as potent anti-inflammatories within the CNS environment, and have been shown to reduce neuro-degeneration and oxidative damage in female mice (Habib & Beyer, 2015), through the direct binding of estradiol to ERα receptors on immune cells (Tanapat et al., 1999; Pyter et al., 2013; Ito et al., 2002). Conversely, testosterone has been shown to suppress immune function and promote pro-degenerative mechanisms (Agostini et al., 2020). Therefore, the research suggests that females may be less susceptible to antibiotic-induced degeneration, due to the interaction of estradiol with immune cells.

Antibiotic-induced increases in pro-inflammatory markers was also associated with disruptions in the behavioural responses to LPS. Our results showed that antibiotic-treated male and female mice displayed significantly less LPS-induced sickness behaviours at eight hours' post-treatment, compared to their water-treated counterparts. This result was inconsistent with our first hypothesis. Sickness behaviors are induced by increased cytokine mRNA expression in thalamic regions (Viviani et al., 2003; Ericsson et al., 1995). Cytokines bind to IL receptors on neurons to induce sickness behaviours (Dantzer, 2004). However, pre-existing neuro-degenerative markers (TNFα mRNA expression) in the antibiotic-treated mice suggests that the neural pathways implicated in sickness behaviours have been disrupted. As previously discussed, chronic hyperactivity of thalamic regions may have increased degenerative markers and neural apoptosis in thalamic regions, thus supressing sickness behaviours. This effect would be particularly impactful during the pubertal period, as the pubertal brain is undergoing significant neural re-organization (Blaustein et al., 2016). However, more research is required to determine if this effect is conserved in later time-points.

4.2. Impact of probiotic consumption on acute immune responsivity

The current thesis showed that probiotic supplementation was associated with significant alterations of the intestinal environment, and LPS-induced inflammation. First, mice supplemented with lacidofil (95% *Lactobacillus rhamnosus* and 5% *Lactobacillus helveticus*) prior to LPS exposure had significantly higher intestinal weights compared to LPS-treated placebo counterparts. Animal studies have also shown that oral supplementation with *Lactobacillus* species have protective effects against LPS-induced disruptions of the intestinal environment (Cui et al., 2017; Murray et al., 2019). For example, one week of treatment with *Lactobacillus reuteri* and *Lactobacillus rhamnosus* in C57BL/6 mice attenuated LPS-induced increases in intestinal permeability, as seen through increased occludin and claudin-3 expression in the intestinal barrier (Cui et al., 2017). Moreover, *Lactobacilli* supplementation in mice reduced LPS-induced cytokine expression and immune cell activation in the intestinal lumen (Shi et al., 2018; Perdigon et al., 1990; Servin, 2004). Therefore, lacidofil supplementation may have reinforced the intestinal barrier and reduced LPS-induced changes in the intestinal environment, thus reducing LPS-induced fluid excretion, diarrhea, and intestinal weight loss.

Probiotic-induced reinforcement of the intestinal environment was also associated with reduced peripheral inflammation in a cytokine-specific manner. In water-treated mice, probio'stick supplementation (90% *Lactobacillus helveticus* and 5% *Bifidobacterium longum*) reduced LPS-induced IL1β and IFNγ plasma concentrations, compared to placebo-treated counterparts; lacidofil supplementation (95% *Lactobacillus rhamnosus* and 5% *Lactobacillus helveticus*) reduced LPS-induced IL1β plasma concentrations compared to placebo-treated counterparts. The results are consistent with our second hypothesis, and with previous research (Murray et al., 2020; Christensen et al., 2002; Elderman et al., 2018; Ferreira dos Santos et al.,

2016; Sichetti et al., 2018). The cytokines IL1β and IFNγ are implicated in pro-inflammatory processes. IL1β is a pro-inflammatory cytokine produced through NLRP3-inflammasome activation (Dinarello, 2018; Garlanda et al., 2013), and is involved in up-regulating NF-κβ-induced pro-inflammatory cytokines (O'Neill, 2008). Similarly, IFN-γ potentiates pro-inflammatory signaling through alterations of NLRP3-inflammasome activity (Kopitar-Jerala, 2017). It is theorized that the probiotic supplementation reduced pro-inflammatory cytokine production through competitive binding with LPS on TLR-expressing immune cells (Servin, 2004). Research has shown that microbe-associated molecular patterns (MAMPs) on the surface of the *Lactobacilli* strains bind to TLR receptors and activate negative regulators (e.g. A20, SOCS1, IRAK3), which suppress nuclear translocation of NF-κβ and ultimately reduce the transcription of pro-inflammatory cytokines (Sun et al., 2017; Vissers et al., 2011). Research has also shown that probiotic species have unique MAMPS on their bacterial cell walls and induce species-specific changes in cytokine production (Cleveland et al., 1996; Christensen et al., 2002; Peña & Versalovic, 2003; Sun et al., 2017). Therefore, slight molecular differences in recognition proteins on *Lactobacillus rhamnosus* and *Lactobacillus helveticus*' bacterial walls would explain why probio'stick (95% *Lactobacillus rhamnosus*) reduced LPS-induced IL1β and IFN-γ concentrations, and lacidofil (90% *Lactobacillus helveticus*) reduced LPS-induced IL1β concentrations.

The reduction in peripheral LPS-induced cytokine expression was also associated with alterations in hippocampal cytokine mRNA expression. Male mice supplemented with lacidofil (95% *Lactobacillus rhamnosus* and 5% *Lactobacillus helveticus*) or probio'stick (90% *Lactobacillus helveticus* and 5% *Bifidobacterium longum*) prior to LPS exposure had significantly lower LPS-induced hippocampal IL-6 mRNA expression, compared to their

placebo-treated counterparts. These results supported our second and fourth hypotheses, and is consistent with previous research (Murray et al., 2020; Christensen et al., 2002; Elderman et al., 2018; Sichetti et al., 2018). Studies from our laboratory have also shown that two weeks of probiotic supplementation significantly reduced hippocampal IL-6, IL1β, and TNFα mRNA expressions after LPS exposure (Murray et al., 2020). The cytokine IL-6 is involved in both pro-inflammatory (JAK/STAT) and anti-inflammatory (MAPK) mechanisms, as well as the regulation of cellular metabolism, regeneration, and neural processes (Diehl & Rincón, 2002; Scheller et al., 2011). It is theorized that reductions in LPS-induced plasma cytokine concentrations in supplemented mice influenced central mRNA expression through humoral and neuronal cytokine signaling routes. Reduced peripheral pro-inflammatory cytokine concentrations would reduce the strength of the inflammatory signals converging into thalamic regions, thus reducing microglial activation and up-regulation of IL-6 mRNA expression, compared to the placebo-treated counterparts.

Notably, the effect of the probiotics on LPS-induced IL-6 mRNA expression was limited to the males. Males exposed to LPS displayed significantly higher IL-6 mRNA expression compared to females, and supplementation with lacidofil and probio'stick significantly reduced IL-6 mRNA expression. Previous research from our laboratory has shown similar sexual dimorphism with other *Lactobacillus* strains on LPS-induced cytokine expression: Supplementation with kefir (containing *L. lactis, L. cremoris, L. diacetylactis,* and *L. acidophilus*) eliminated LPS-induced IL-6 mRNA expression in the hippocampus and hypothalamus of pubertal males. In females, the supplementation reduced LPS-induced prefrontal cortex IL-1β mRNA expression, and hippocampal TNFα mRNA expression (Murray et al., 2019). The sexually dimorphic effect of probiotics on central inflammation may be due to

differences in the microbiome during the pubertal period. In pubertal NOD/ShiLtJ mice, pubertal onset in males is associated with reduced microbial diversity, while pubertal females maintain higher microbial diversity into adulthood (Yurkovetskiy et al., 2013; Jašarević et al., 2016; Org et al., 2016). Research in BALB/c and C57BL/6 mice has also shown that bacterial strains such as *Lactobacillus plantarum*, *Bacteroides distasonis*, and *Bifidobacterium* were higher in females compared to males (Elderman, Hugenholtz, et al., 2018). Therefore, probiotic supplementation may confer more benefits in males, due to their reduced microbial diversity and lower proportions of *Lactobacillus* and *Bifidobacterium* strains.

4.3. Mitigation of antibiotic-induced changes on acute immune responsivity by probiotic supplementation

The current thesis showed that lacidofil supplementation altered antibiotic-induced changes in LPS-induced inflammation and sickness behaviours. This study found that lacidofil supplementation (95% *Lactobacillus rhamnosus* and 5% *Lactobacillus helveticus*) in antibiotic-treated mice significantly reduced LPS-induced IL-12 and IFNγ plasma concentrations compared to placebo-treated mice. The results are consistent with our third hypothesis, as well as with previous research. Previous research has also shown that specific *lactobacillus* strains are particularly efficient at altering stress-induced pro-inflammatory cytokine expression (Vissers et al., 2011; Christensen et al., 2002; Perdigon et al., 1990; Jang et al., 2018; Laval et al., 2015). It is theorized that *Lactobacillus rhamnosus* is uniquely efficient at suppressing antibiotic-induced inflammation, by competitively binding with bacterial endotoxins and suppressing pro-inflammatory processes (Frick et al., 2007; Sichetti et al., 2018; Villena & Kitazawa, 2014). Due to unique recognition proteins of *Lactobacillus rhamnosus*, lacidofil supplementation may be

more suited for competitive binding in stressed systems of pubertal mice when compared to the recognition proteins of *Lactobacillus helveticus* (probio'stick). It is noted that the mechanisms behind these effects have yet to be elucidated, and more research is required to determine how these probiotic bacteria function in stressed systems.

Alterations of antibiotic-induced peripheral inflammation by lacidofil was also associated with reductions in sickness behaviours. The results showed that regardless of LPS or saline treatment, antibiotic-treated mice supplemented with lacidofil (95% *Lactobacillus rhamnosus* and 5% *Lactobacillus helveticus*) displayed significantly less sickness behaviours compared to placebo and probio'stick treated counterparts. The result was partially consistent with our third hypothesis and with previous research. Research from our laboratory has shown that two weeks of probiotic supplementation with *Lactobacillus reuteri* reduced LPS-induced sickness behaviours (Murray et al., 2020). It is theorized that the reduction of peripheral cytokine concentrations in antibiotic-treated mice exposed to lacidofil reduced the convergence of inflammatory signals to the lower brain regions, thus reducing cytokine-induced neural activation of thalamic regions implicated in sickness behaviours.

4.4. Limitations and future directions

Neuromicrobiology is a relatively new field. Despite a surge of research in the past decade, the common methods used to induce microbial dysbiosis have several caveats. Firstly, the microbiome interacts with multiple intricate systems to maintain symbiosis and homeostasis. Disrupting the microbiome induces changes in metabolic function, endocrine function, structure and anatomy, immune function, sexual development, brain function, and more (Kennedy et al., 2018; Clarke et al., 2014; Dinan & Cryan, 2017; Desbonnet et al., 2015). As such, it is likely that

our treatments (antibiotics and probiotics) influenced multiple systems beyond the immune system, which makes it difficult to accurately interpret the mechanisms underlying the observed effects. Further research using our treatment model is required to identify changes occurring across multiple systems, and to determine if these alterations align with our interpretations of the observed effects.

Secondly, there are ongoing debates about whether the gavage treatment is stressful to mice, particularly pubertal mice. Studies have shown that a single gavage in rats elevated plasma corticosterone levels four hours post-treatment (Brown et al., 2000). As well, a week of gavage in male C57BL/6 mice increased blood pressure and heart rate five hours after treatment, and increased fecal corticosterone metabolite concentrations (Walker et al., 2012). However, other researchers have found no significant difference in stress measures. For example, female C57BL/6 mice that underwent 18 days of gavage showed no difference in plasma corticosterone levels at 24 hours post-treatment, compared to controls (Jones et al., 2016); male and female CD-1 mice that underwent six weeks of gavage treatments also showed no difference in plasma corticosterone at the time of euthanasia (24 hours after last gavage treatment; Arantes-Rodrigues et al., 2012). In the current study, considering that the gavage treatments were administered during a critical period of development (puberty), there may have been stress-induced changes in the developmental trajectory of our mice. Previous research has shown that restraint stress during puberty induces long-term changes in brain function and behaviours (Barha et al., 2011; Deng et al., 2017; Huo et al., 2017). Further research with our gut dysbiosis model should consider a control non-gavage group to determine if there are significant effects of the gavage on immune responsivity.

In relation to the study itself, there are two identified caveats. The first caveat is the acute nature of the study. All tissues were collected at 8 hours' post-injection in six-week old mice. As such, this study is purely mechanistic, and cannot be used to extrapolate brain function and behaviour outside of this timeframe. Further research is required to assess the long-term effects of pubertal microbial shifts on brain function and behaviour. The second caveat is the sample size used for the tissue analyses. It is standard in our laboratory to use a sample size of 5 per group for rt-qPCR. However, with the large number of groups (24 groups), this sample size may have reduced the power of our statistical analyses. Larger sample sizes are suggested for further studies with large group numbers.

4.5. Conclusions

The aim of this study was to determine if pubertal microbial dysbiosis would influence acute immune responsivity in male and female mice, and if this effect could be mitigated by probiotics. Based on the analyses, it can be concluded that gut dysbiosis aggravated the pro-inflammatory immune response in a sex-specific manner. This was associated with increased neural degeneration, which was theorized to have disrupted neural circuitry and suppressed sickness behaviours. As well, probiotic supplementation reduced the pro-inflammatory immune response in a sex-specific manner. Depending on the status of the microbiome, supplementation with different strains of bacteria had unique effects on the acute immune response. Lacidofil supplementation (mostly composed of *Lactobacillus rhamnosus*) was more effective at reducing the inflammatory response in pubertal mice experiencing gut dysbiosis; probio'stick supplementation (mostly composed of *Lactobacillus helveticus*) was more effective in pubertal mice with an unaltered microbiome. This was one of the first studies to assess the effects of

pubertal microbial dysbiosis on the acute immune response. Overall, we found a potential mechanism for the behavioural dysfunctions associated with adolescent gut dysbiosis (such as irritable bowel syndrome). As well, we have shown that probiotic strains—such as *Lactobacillus rhamnosus*—are effective for therapeutic interventions in pubertal individuals suffering from gut microbial dysfunctions.

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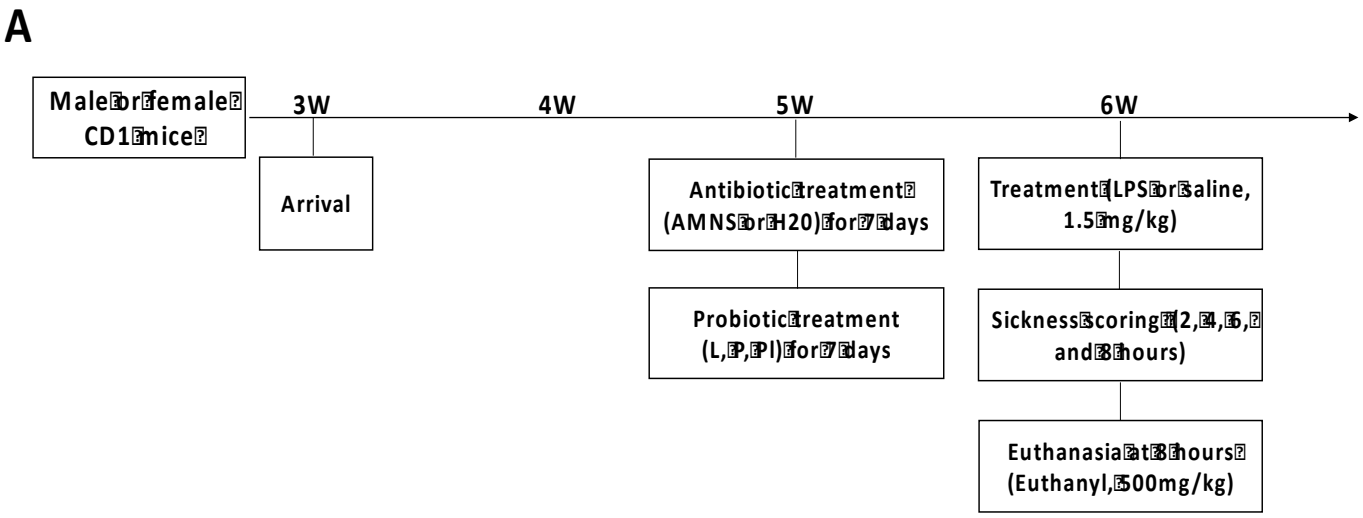

A
Male or female CD1 mice
3W
4W
5W
6W
Arrival
Antibiotic treatment (AMNS or H20) for 7 days
Probiotic treatment (L, P, Pl) for 7 days
Treatment (LPS or saline, 1.5 mg/kg)
Sickness Scoring (2, 4, 6, and 8 hours)
Euthanasia at 8 hours (Euthanyl, 500mg/kg)

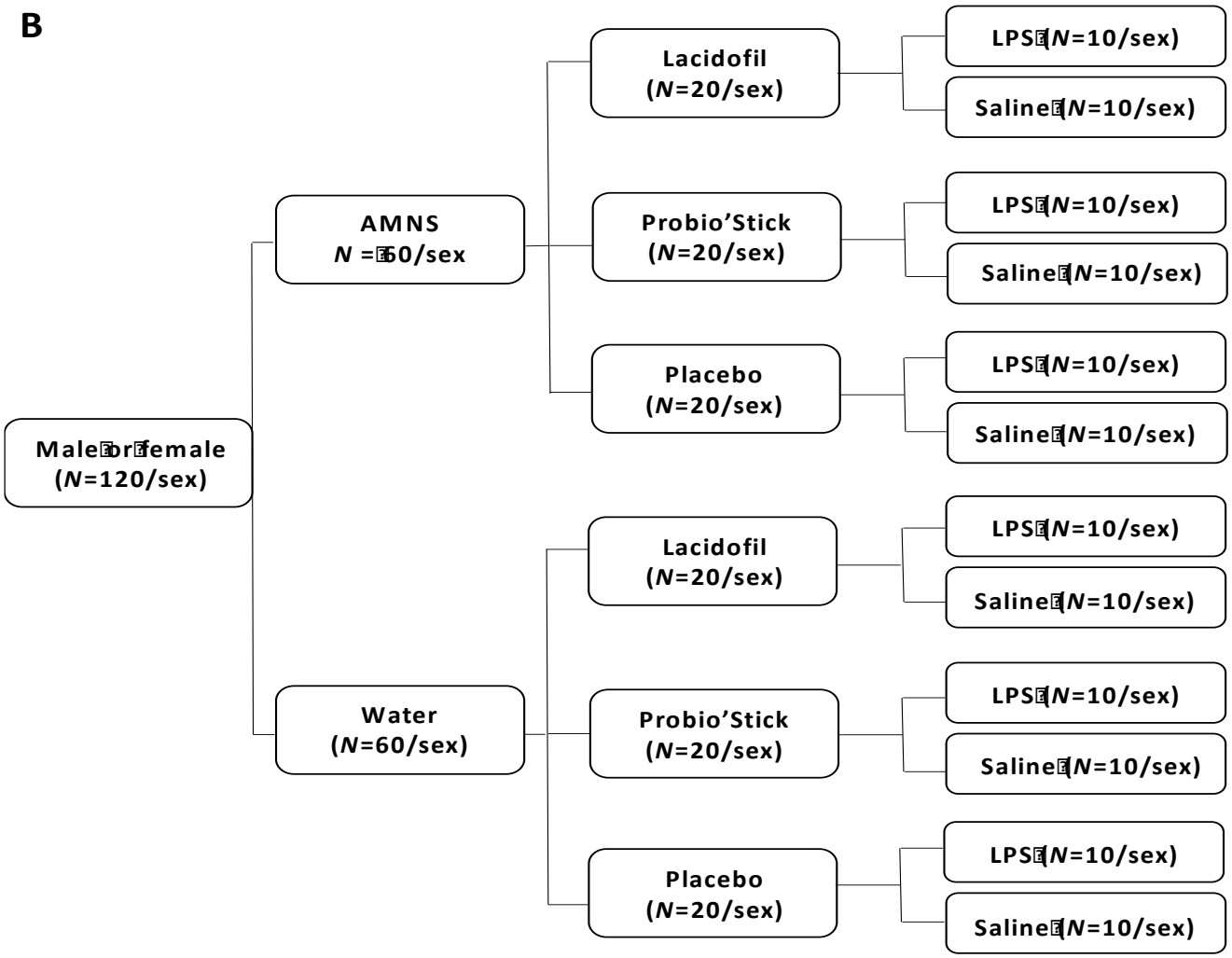
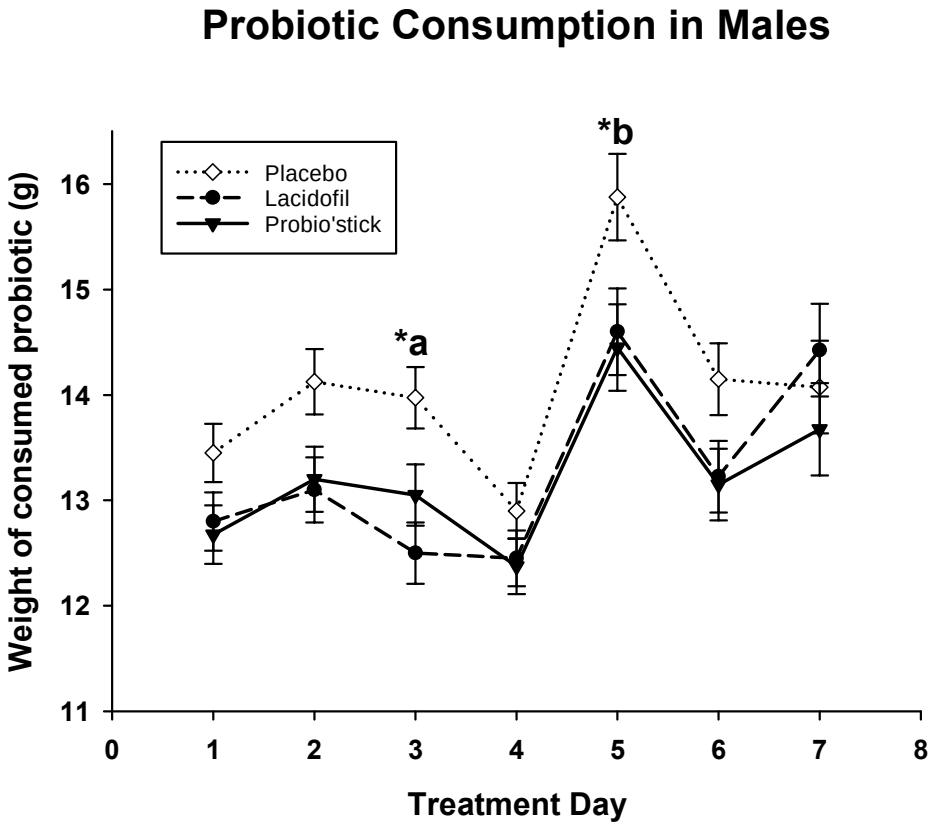

B
Male or female (N=120/sex)
AMNS N = 60/sex
Water (N=60/sex)
Lacidofil (N=20/sex)
Probio'Stick (N=20/sex)
Placebo (N=20/sex)
Lacidofil (N=20/sex)
Probio'Stick (N=20/sex)
Placebo (N=20/sex)
LPS (N=10/sex)
Saline (N=10/sex)
LPS (N=10/sex)
Saline (N=10/sex)
LPS (N=10/sex)
Saline (N=10/sex)
LPS (N=10/sex)
Saline (N=10/sex)
LPS (N=10/sex)
Saline (N=10/sex)
LPS (N=10/sex)
Saline (N=10/sex)

Figure 1.

A


Probiotic Consumption in Males
Placebo
Lacidofil
Probio'stick
*a
*b
Weight of consumed probiotic (g)
Treatment Day
11
12
13
14
15
16
0
1
2
3
4
5
6
7
8

B

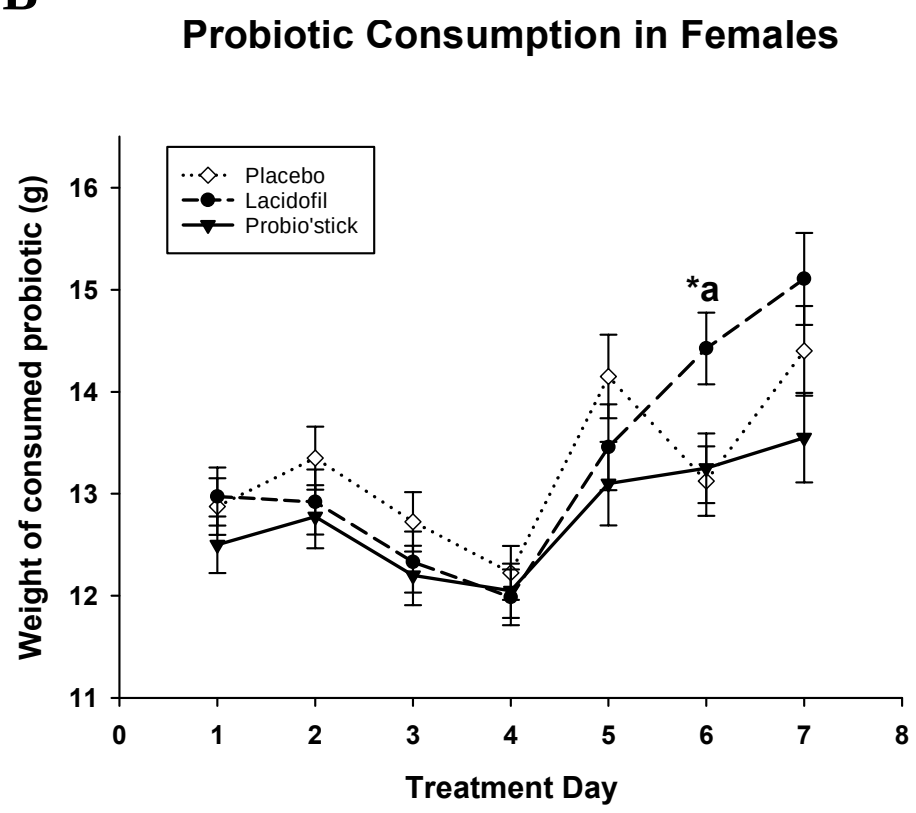
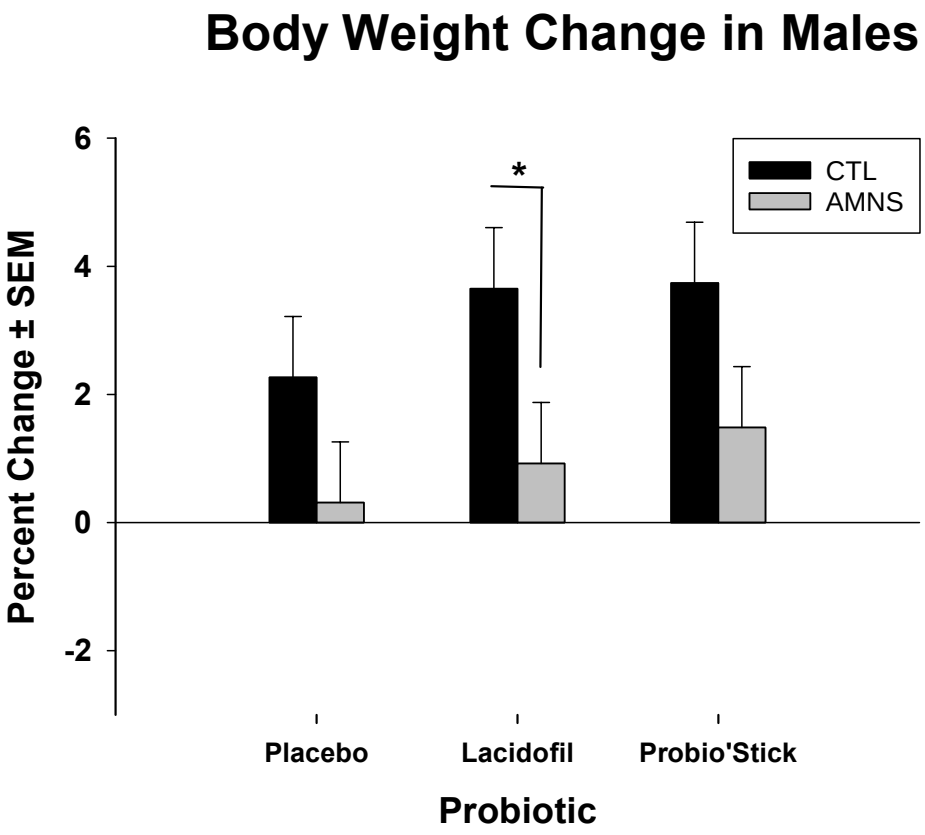

Probiotic Consumption in Females
Placebo
Lacidofil
Probio'stick
*a
Weight of consumed probiotic (g)
Treatment Day
11
12
13
14
15
16
0
1
2
3
4
5
6
7
8

Figure 2.

A


Body Weight Change in Males
Percent Change ± SEM
6
4
2
0
-2
*
CTL
AMNS
Placebo
Lacidofil
Probio'Stick
Probiotic

B

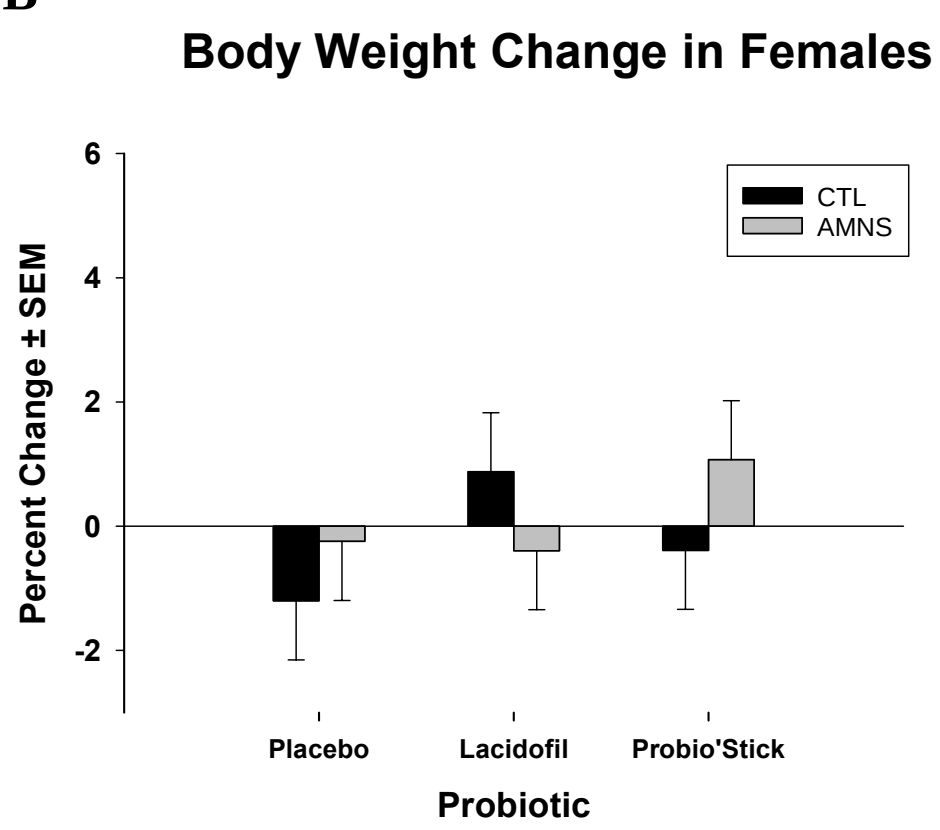
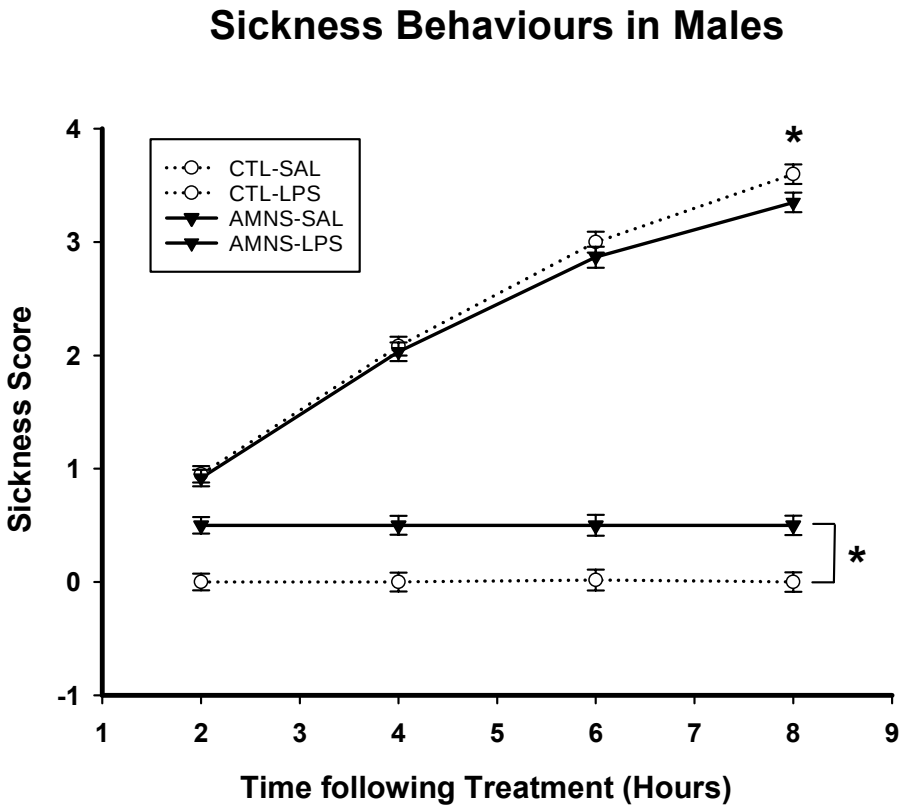

Body Weight Change in Females
Percent Change ± SEM
6
4
2
0
-2
CTL
AMNS
Placebo
Lacidofil
Probio'Stick
Probiotic

Figure 3.

A


Sickness Behaviours in Males
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
Sickness Score
Time following Treatment (Hours)
*
*

B

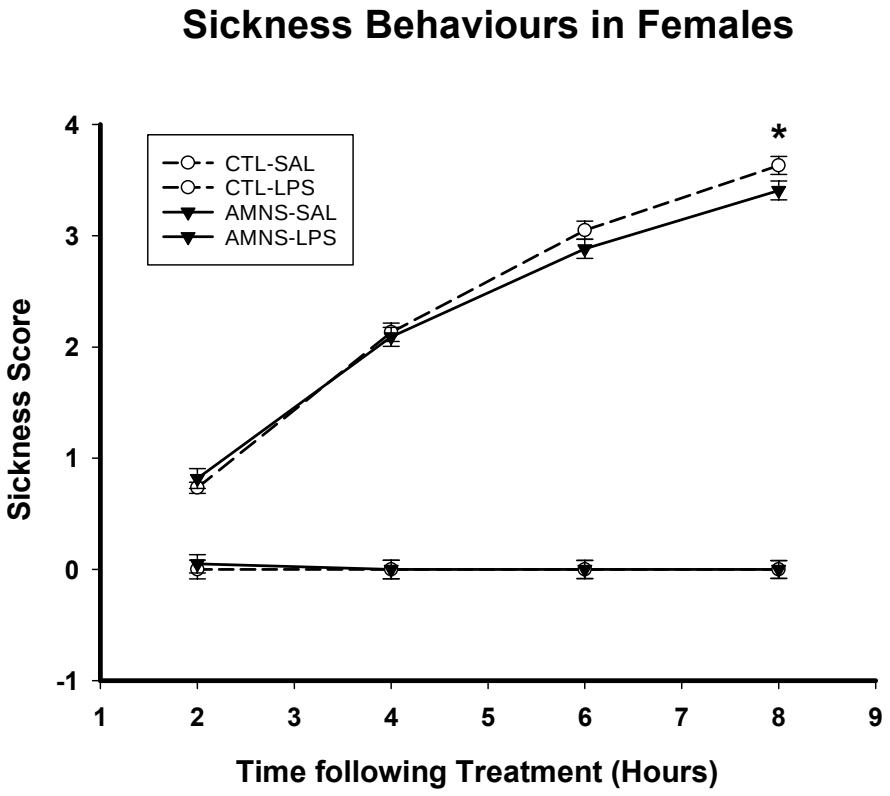
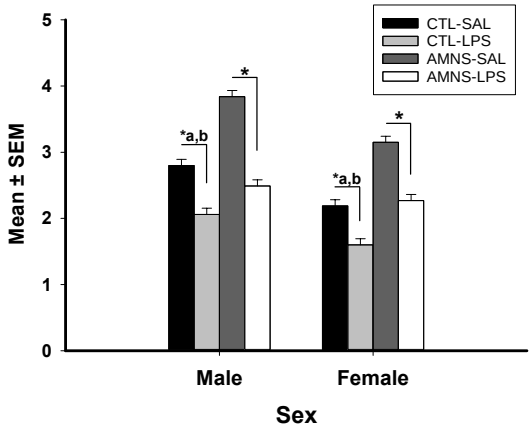

Sickness Behaviours in Females
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
Sickness Score
Time following Treatment (Hours)
*

Figure 4.

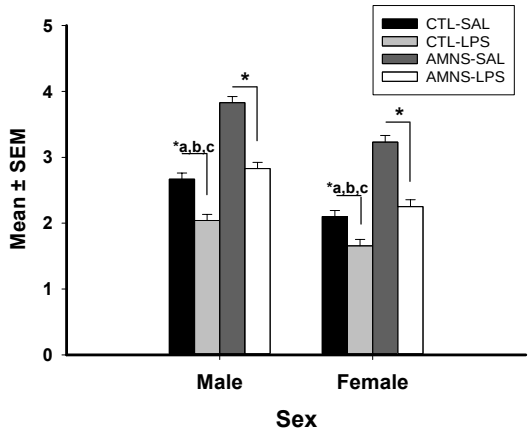
A

Whole Intestine Weights of Placebo-Treated Mice



B

Whole Intestine Weights of Lacidofil-Treated Mice



C

Whole Intestine Weights of Probio'stick-Treated Mice

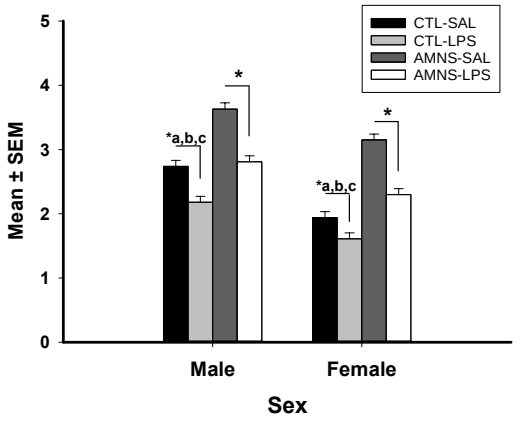
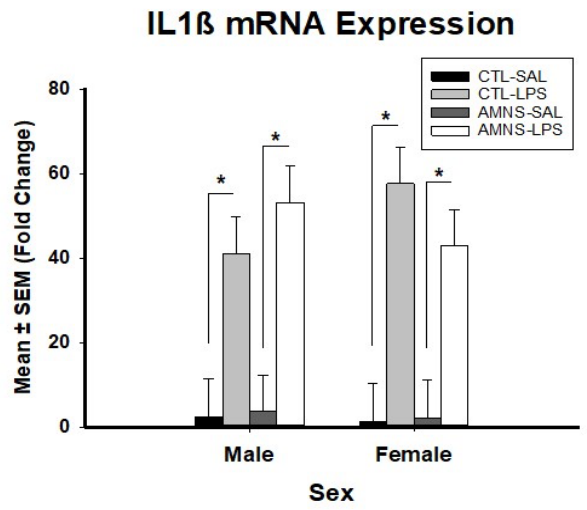
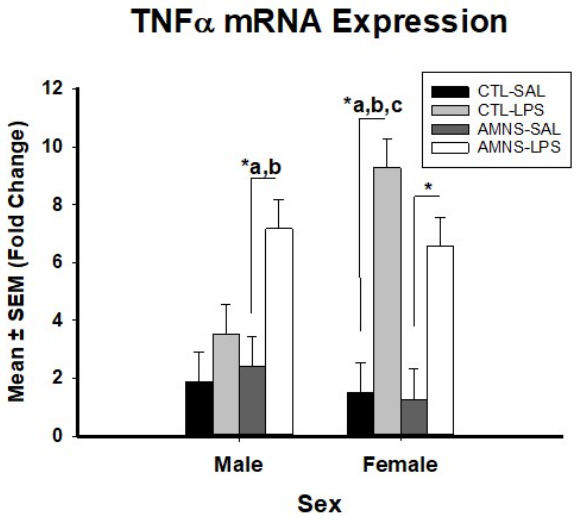


Figure 5.

A


IL1ß mRNA Expression
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
Mean ± SEM (Fold Change)
80
60
40
20
0
*
*
*
*
Male
Female
Sex

B


TNFα mRNA Expression
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
Mean ± SEM (Fold Change)
12
10
8
6
4
2
0
*a,b
*a,b,c
*
Male
Female
Sex

C

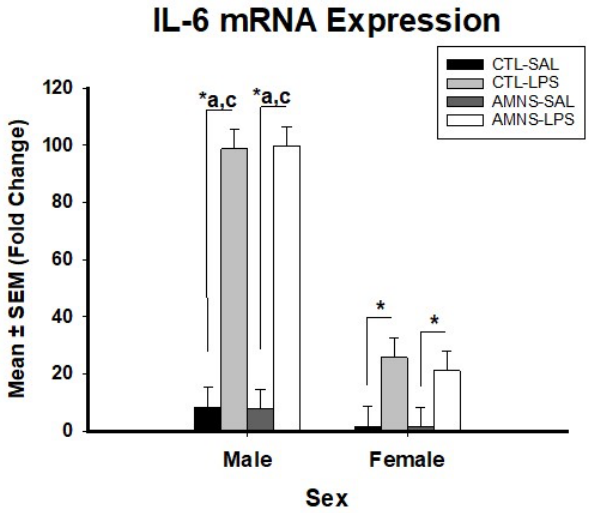
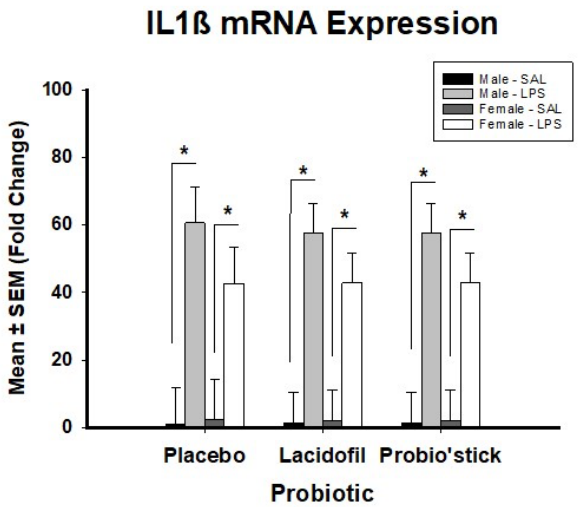
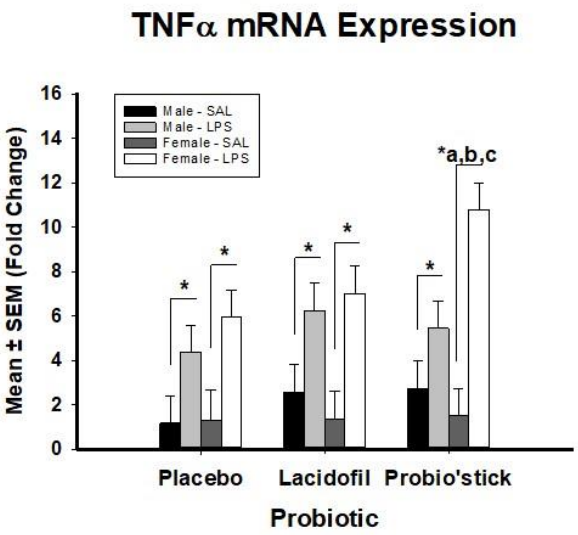

IL-6 mRNA Expression
CTL-SAL
CTL-LPS
AMNS-SAL
AMNS-LPS
Mean ± SEM (Fold Change)
120
100
80
60
40
20
0
*a,c
*a,c
*
*
Male
Female
Sex

Figure 6.

A


IL1ß mRNA Expression
Male - SAL
Male - LPS
Female - SAL
Female - LPS
Mean ± SEM (Fold Change)
100
80
60
40
20
0
*
Placebo
Lacidofil
Probio'stick
Probiotic

B


TNFα mRNA Expression
Male - SAL
Male - LPS
Female - SAL
Female - LPS
Mean ± SEM (Fold Change)
16
14
12
10
8
6
4
2
0
*
*a,b,c
Placebo
Lacidofil
Probio'stick
Probiotic

C

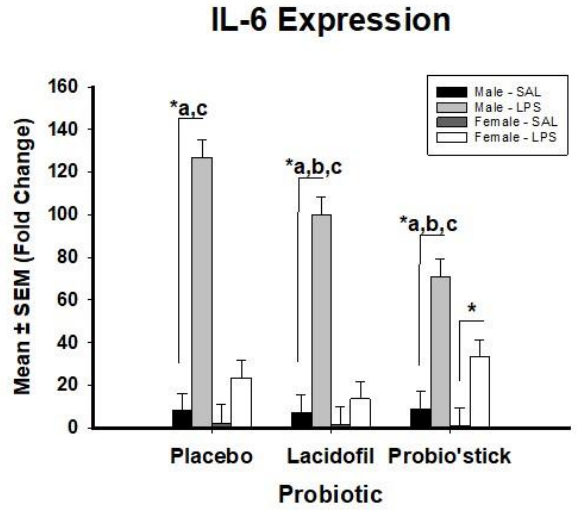
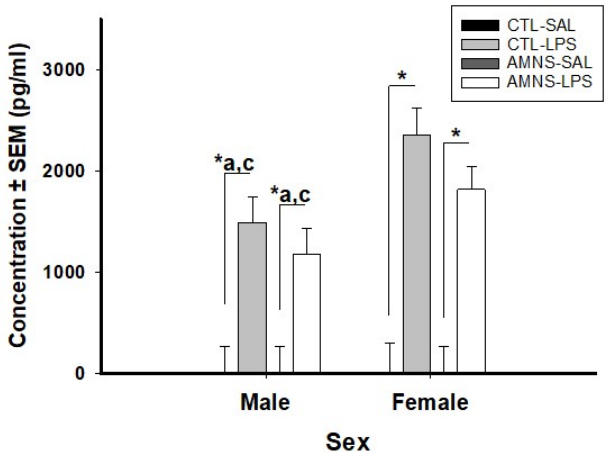

IL-6 Expression
Male - SAL
Male - LPS
Female - SAL
Female - LPS
Mean ± SEM (Fold Change)
160
140
120
100
80
60
40
20
0
*a,c
*a,b,c
*a,b,c
*
Placebo
Lacidofil
Probio'stick
Probiotic

Figure 7.

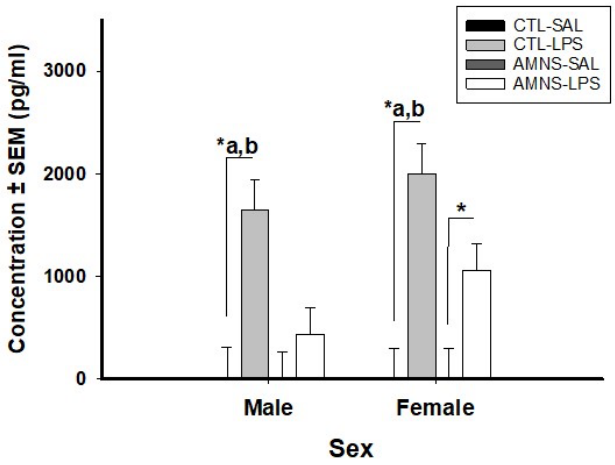
A

IFNψ Plasma Concentration in Placebo-Treated Mice



B

IFNψ Plasma Concentration in Lacidofil-Treated Mice



C

IFNψ Plasma Concentration in Probio'stick-Treated Mice

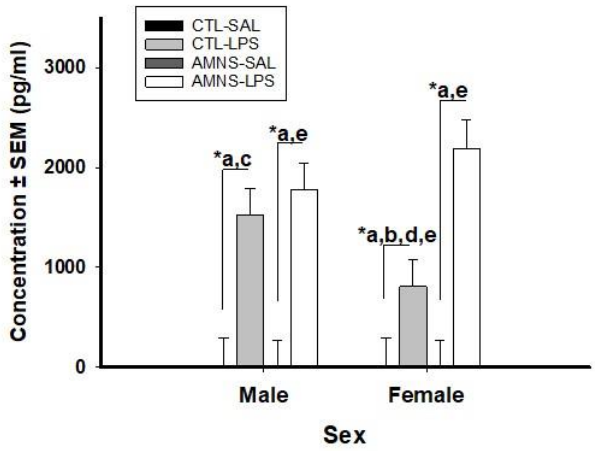
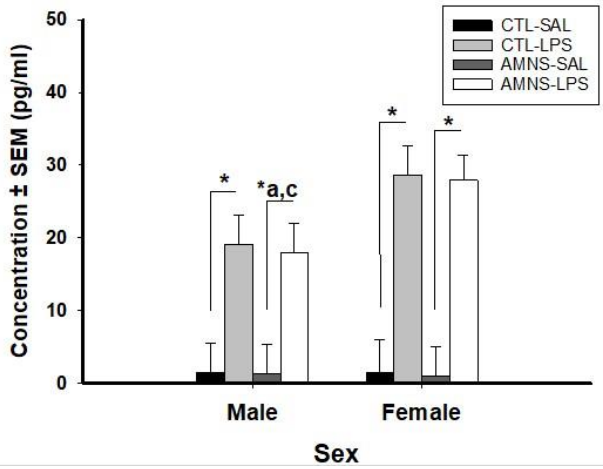


Figure 8.

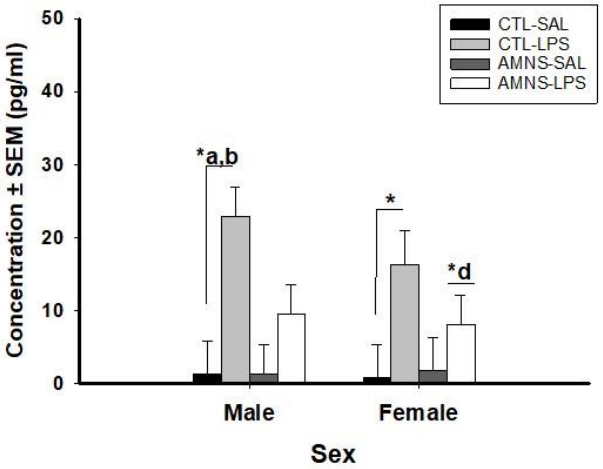
A

IL-1ß Plasma Concentration in Placebo-Treated Mice



B

IL-1ß Plasma Concentration in Lacidofil-Treated Mice



C

IL-1ß Plasma Concentration in Probio'stick-Treated Mice

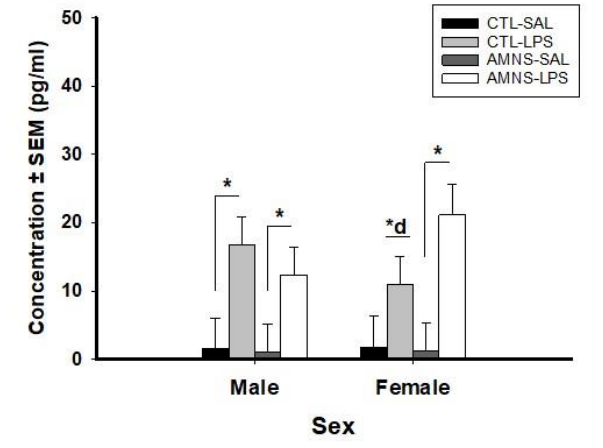
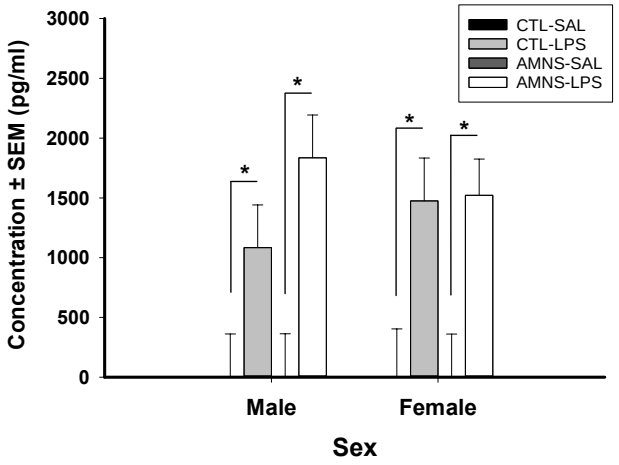


Figure 9.

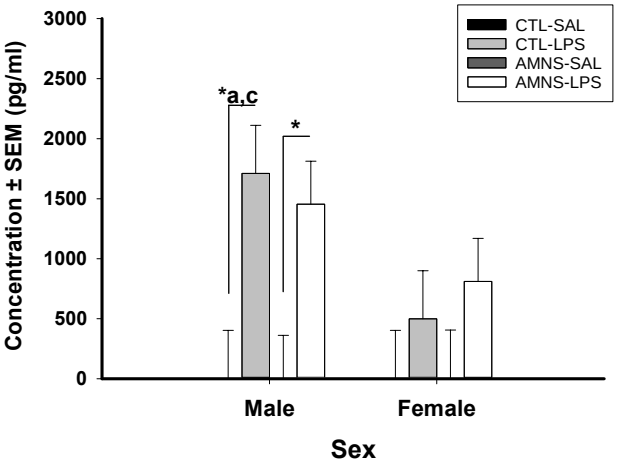
A

IL-6 Plasma Concentration in Placebo-Treated Mice



B

IL-6 Plasma Concentration in Lacidofil-Treated Mice



C

IL-6 Plasma Concentration in Probio'stick-Treated Mice

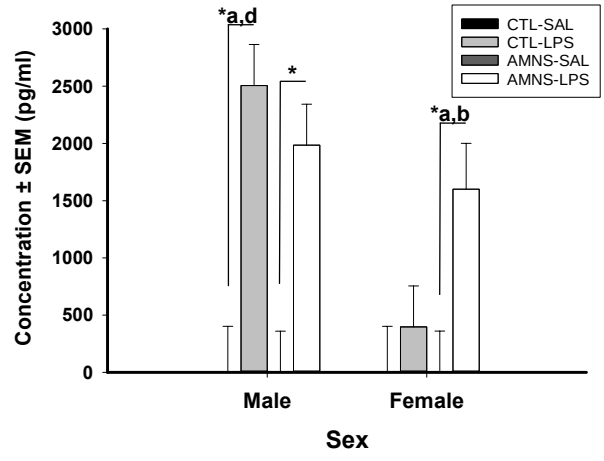
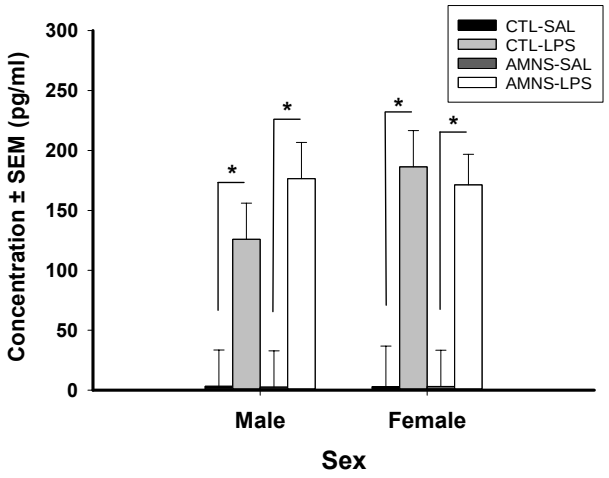
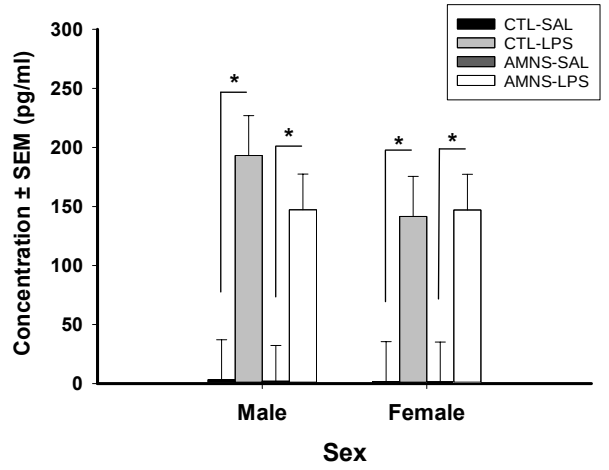


Figure 10.

A



B



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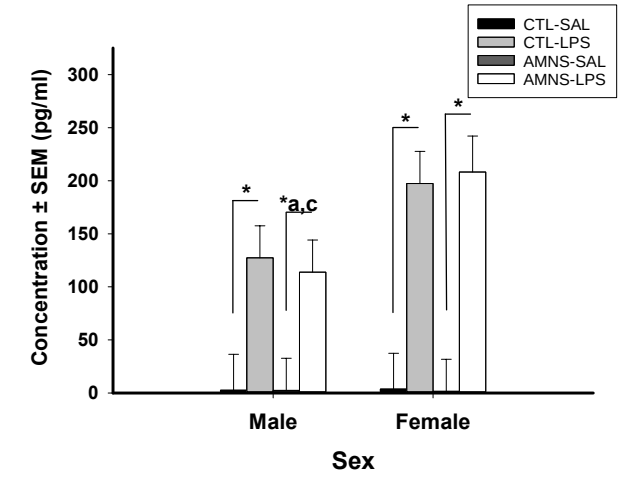
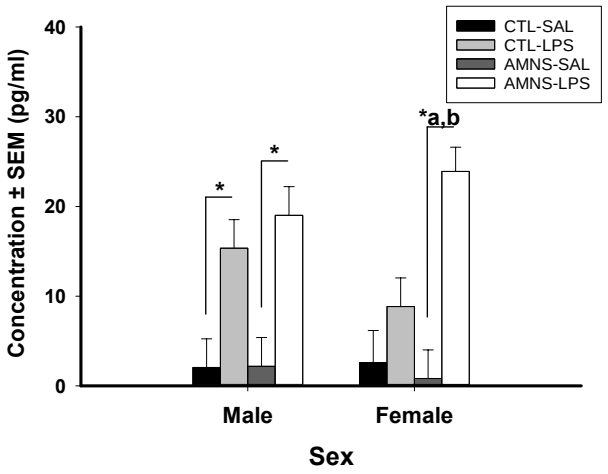


Figure 11.

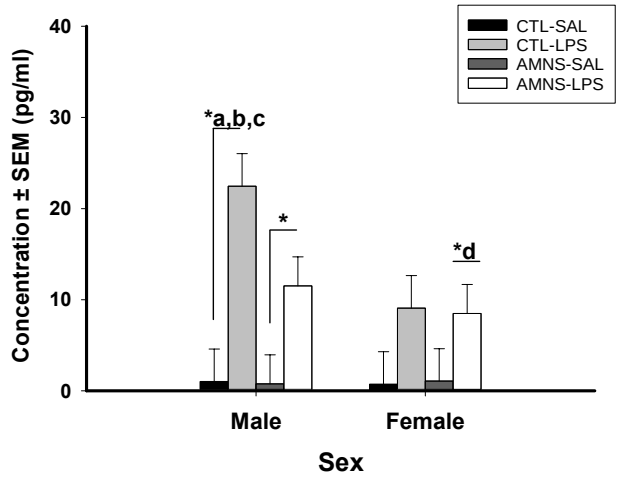
A

IL-12 Plasma Concentration in Placebo-Treated Mice



B

IL-12 Plasma Concentration in Lacidofil-Treated Mice



C

IL-12 Plasma Concentration in Probio'stick-Treated Mice

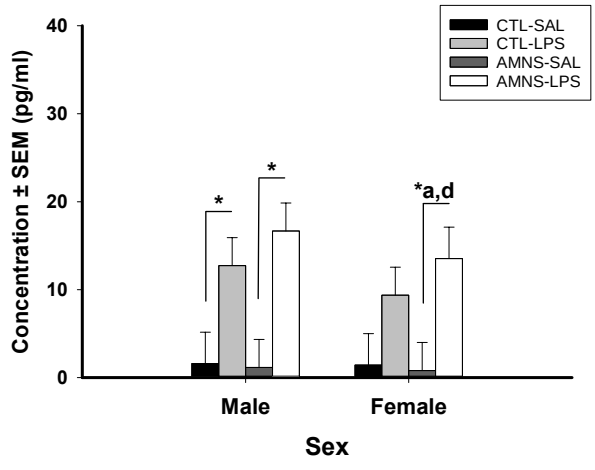
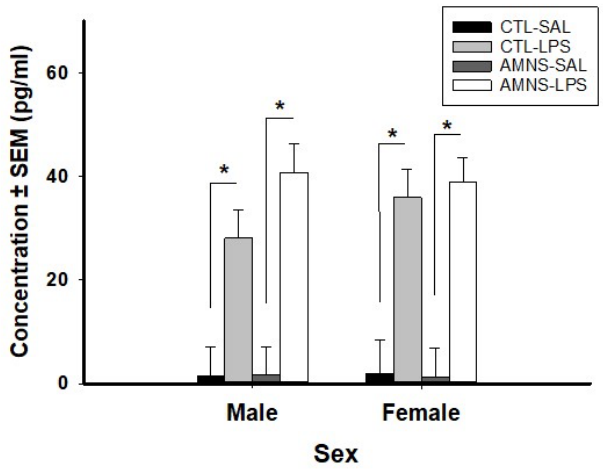


Figure 12.

A



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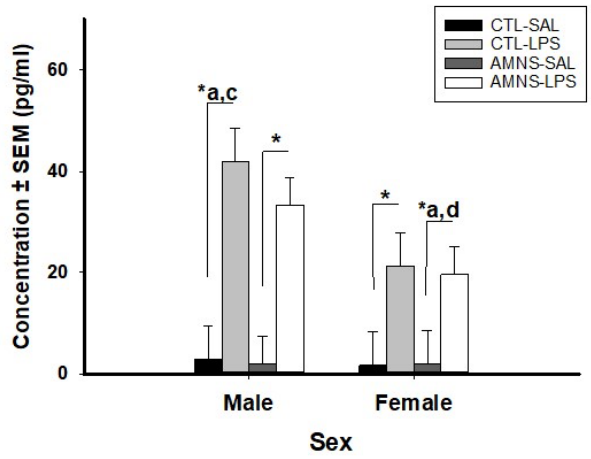

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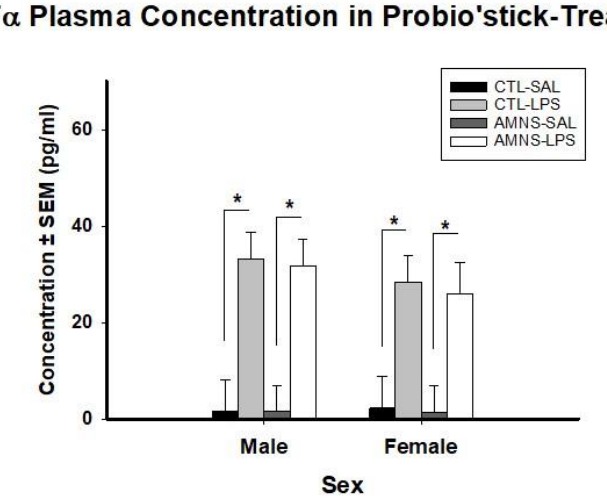

Figure 13.

Figure Captions

Figure 1. Experimental timeline and experimental groups for six-week old male and female mice. *Note:* AMNS = Ampicillin, metronidazole, neomycin, and streptomycin (antibiotics); L = Lacidofil, P = Probio'Stick, Pl = Placebo, LPS = lipopolysaccharide.

Figure 2. Daily consumption volume of six-week old (A) male and (B) female mice supplemented with placebo, lacidofil, or probio'stick. Data represented as mean consumption (±SEM), n = 78-80/group. The asterisks (*a) denotes a significant difference between placebo and lacidofil treatments ($p < 0.05$), (*b) denotes a significant difference between placebo and probio'stick treatments ($p < 0.05$).

Figure 3. Percent body weight change in six-week old (A) male and (B) female mice treated with water (CTL) or antibiotics (AMNS) and supplemented with either placebo, lacidofil, or probio'stick. Data represented as a mean percentage (±SEM), n = 18-20/group. (*) denotes a significant difference between antibiotic-treated and water-treated mice ($p < 0.05$).

Figure 4. Acute sickness scores of six-week old (A) male and (B) female mice treated with saline (SAL) or lipopolysaccharide (LPS) and treated with either water (CTL-SAL, CTL-LPS) or antibiotics (AMNS-SAL, AMNS-LPS). Data represented as mean sickness scores (±SEM), n = 28-30/group. (*) denotes a significant difference between antibiotic and water-treated mice ($p < 0.05$).

Figure 5. Whole intestinal weights of (A) placebo-treated, (B) lacidofil-treated and (C) probio'stick-treated six-week old male and female mice treated with saline (SAL) or lipopolysaccharide (LPS) and treated with either water (CTL-SAL, CTL-LPS) or antibiotics (AMNS-SAL, AMNS-LPS). Data represented as mean (±SEM), n = 8-10/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (*b) denotes a significant difference between antibiotic and water-treated counterparts ($p < 0.05$), and (*c) denotes a significant difference between male and female counterparts.

Figure 6. Acute hippocampal (A) IL-1β, (B) TNFα, and (C) IL-6 mRNA expression in six-week old male and female mice treated with either saline (SAL) or lipopolysaccharide (LPS) and with either water (CTL-SAL, CTL-LPS) or antibiotics (AMNS-SAL, AMNS-LPS). Data represented as mean fold change (±SEM), n = 12-15/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (*b) denotes a significant difference between antibiotic and water-treated counterparts ($p < 0.05$).

Figure 7. Acute hippocampal (A) IL-1β, (B) TNFα, and (C) IL-6 mRNA expression in six-week old male and female mice treated with saline (SAL) or lipopolysaccharide (LPS) and with either placebo, lacidofil, or probio'stick. Data represented as mean fold change (±SEM), n = 18-20/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (*b) denotes a significant difference between the probiotic and the placebo counterpart ($p < 0.05$), and (*c) denotes a significant difference between male and female counterparts.

Figure 8. Acute plasma IFNγ concentration in (A) placebo-treated, (B) lacidofil-treated and (C) probio'stick-treated six-week old male and female mice treated with either saline (SAL) or lipopolysaccharide (LPS) and with either water (CTL-SAL, CTL-LPS) or antibiotics (AMNS-SAL, AMNS-LPS). Data represented as mean concentration (±SEM), n = 4-5/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (*b) denotes a significant difference between antibiotic and water-treated counterparts ($p < 0.05$), (*c) denotes a significant difference between male and female counterparts, (*d) denotes a significant difference between the probiotic group and the placebo counterpart, and (*e) denotes a significant difference between lacidofil and probio'stick counterparts.

Figure 9. Acute plasma IL-1β concentration in (A) placebo-treated, (B) lacidofil-treated and (C) probio'stick-treated six-week old male and female mice treated with either saline (SAL) or lipopolysaccharide (LPS) and with either water (CTL-SAL, CTL-LPS) or antibiotics (AMNS-SAL, AMNS-LPS). Data represented as mean concentrations (±SEM), n = 4-5/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (*b) denotes a significant difference between antibiotic- and water-treated counterparts ($p < 0.05$), (*c) denotes a significant difference between male and female counterparts, and (*d) denotes a significant difference between the probiotic group and the placebo counterparts.

Figure 10. Acute plasma IL-6 concentration in (A) placebo-treated, (B) lacidofil-treated and (C) probio'stick-treated six-week old male and female mice treated with either saline (SAL) or lipopolysaccharide (LPS) and with either water (CTL-SAL, CTL-LPS) or antibiotics (AMNS-SAL, AMNS-LPS). Data represented as mean concentration (±SEM), n = 4-5/group. The

asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (*b) denotes a significant difference between antibiotic- and water-treated counterparts ($p < 0.05$), (*c) denotes a significant difference between male and female counterparts, and (*d) denotes a significant difference between the probiotic group and the placebo counterpart.

Figure 11. Acute plasma IL-10 in (A) placebo-treated, (B) lacidofil-treated and (C) probio'stick-treated six-week old male and female mice treated with either saline (SAL) or lipopolysaccharide (LPS) and with either water (CTL-SAL, CTL-LPS) or antibiotics (AMNS-SAL, AMNS-LPS). Data represented as mean concentration (±SEM), n = 4-5/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (*b) denotes a significant difference between antibiotic- and water-treated counterparts ($p < 0.05$), and (*c) denotes a significant difference between male and female counterparts.

Figure 12. Acute plasma IL-12 in (A) placebo-treated, (B) lacidofil-treated and (C) probio'stick-treated six-week old male and female mice treated with either saline (SAL) or lipopolysaccharide (LPS) and with either water (CTL-SAL, CTL-LPS) or antibiotics (AMNS-SAL, AMNS-LPS). Data represented as mean concentration (±SEM), n = 4-5/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (*b) denotes a significant difference between antibiotic- and water-treated counterparts ($p < 0.05$), (*c) denotes a significant difference between male and female counterparts, and (*d) denotes a significant difference between the probiotic group and the placebo counterpart.

Figure 13. Acute plasma TNFα in (A) placebo-treated, (B) lacidofil-treated and (C) probio'stick-treated six-week old male and female mice treated with either saline (SAL) or lipopolysaccharide (LPS) and with either water (CTL-SAL, CTL-LPS) or antibiotics (AMNS-SAL, AMNS-LPS). Data represented as mean concentration (±SEM), n = 4-5/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (*b) denotes a significant difference between antibiotic- and water-treated counterparts ($p < 0.05$), and (*c) denotes a significant difference between male and female counterparts, and (*d) denotes a significant difference between the probiotic group and the placebo counterpart.