The effects of probiotic treatment during puberty on LPS-induced immune response in male and female mice

ATIQA PIRWANI, EMMA MURRAY, AND NAFISSA ISMAIL

UNIVERSITY OF OTTAWA

Puberty is a critical developmental period that is particularly vulnerable to stress and inflammation. In mice, exposure to an immune challenge (lipopolysaccharide; LPS) during puberty causes enduring effects on depression- and anxiety-like behaviour into adulthood. While the mechanisms underlying these effects remain unknown, the gut microbiome could play a role in mediating the immune system and can alter brain functioning. Thus, we investigated if colonizing the gut with beneficial microbes via probiotics could mediate the inflammatory response to pubertal LPS treatment, in 80 male and female CD1 mice. Sickness behaviour and pro-inflammatory cytokine mRNA expression via RT-qPCR were examined. LPS treatment increased sickness and inflammation in all mice. However, LPS-treated males showed more sickness behaviour, but less central cytokine mRNA expression compared to females and their control saline-treated counterparts. These effects were eliminated when the mice were treated with probiotics. In females, probiotic treatment reduced sickness behaviour, in a time-specific manner, and reduced cytokine mRNA expression in a region-specific manner following LPS treatment. Our results show that probiotics mitigate the LPS-induced immune response differently between males and females. These findings suggest that probiotics have a protective effect during puberty and may prevent the onset of mental health conditions like depression and anxiety.

Keywords: Stress, Kefir, Inflammation, Sickness Behaviour, RT-qPCR, Brain and Behaviour

Puberty: A critical period of development

Puberty is a developmental period that marks the transition from a non-reproductive state to a reproductive state, resulting in sexual maturity (Sisk & Foster, 2004). During this period, there is also rapid brain remodeling and reorganization (Levitt, 2003). These rapid and complex changes within the central nervous system (CNS) render puberty particularly vulnerable to exposure to stress and immune challenges (Holder & Blaustein, 2014; Kane & Ismail, 2017). More specifically, this exposure can have long-lasting effects on physical and psychological aspects of health, including increased susceptibility to mental illness, such as depression and anxiety (Queen et al., 2016; Holder & Blaustein, 2014). In rodent models, exposure to a variety of stressors, such as heat (Paris et al., 1973), immobilization (Paris et al., 1973) or shipping stress (Laroche et al., 2009) during the pubertal period results in long-term negative effects on reproductive capacity into adulthood. Moreover, social instability stress during adolescence increases anxiety-like behaviour and decreases social interaction in adult male rats (Green et al., 2012). Exposure to an immune challenge during puberty, like lipopolysaccharide (LPS), causes enduring reproductive effects such as reduced sexual receptivity and behavioural responsiveness to hormonal treatments in adulthood (Laroche et al., 2009). LPS may also influence non-reproductive effects including depression- (Ismail & Blaustein, 2013), anxiety- (Olesen et al., 2011), and Parkinson-like behaviour (Girard-Joyal & Ismail, 2017), as well as cognitive function in mice (Ismail & Blaustein, 2013). Exposure to LPS in female mice during puberty alters the behavioural response to ovarian hormones that would normally reduce anxiety-like and depression-like behaviour into adulthood (Olesen et al., 2011). These enduring effects of LPS are limited to the stress-sensitive pubertal period at 6 weeks of age in mice, as exposure to an immune challenge at ages younger or older than 6 weeks do not result in these enduring behavioural alterations.

Disruption of the Immune System via an Immune Challenge

LPS is a constituent of the outer membrane of gram-negative bacteria, which elicits an instant immune response that can be measured at both the molecular and behavioural levels (Kentner & Pittman, 2010; Kolmogorova et al., 2017). Molecularly, there are two types of cytokines that are produced in response to LPS: pro-and anti-inflammatory cytokines. Pro-inflammatory cytokines promote inflammation and sickness behaviour (IL-1β, IL-6 and TNFα), while anti-inflammatory cytokines (IL-4, IL-10) limit inflammation and sickness behaviour (Vilcek, 1998; Bluthé et al., 1999; Leon et al., 1999). Additionally, recent studies have shown that there are sex and age differences in the immune response to infections. Males display greater sickness behaviour at 30 minutes after LPS treatment in comparison to their female counterparts (Cai et al., 2016). Males also display higher levels of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) at 2 hours following LPS treatment in comparison to females (Sharma et al., 2018). However, adult female mice display the greatest increase in corticosterone (CORT) levels two hours following LPS treatment (Girard-Joyal et al., 2015). Age differences were also observed; pubertal mice seem to be more responsive when exposed to an acute stressor (physical or psychological), resulting in a longer hormonal stress response compared to adults (Goldman et al., 1973; Romeo et al., 2004; Vazquez & Akil, 1993). Age differences that occur in the stress response can be a result of the different levels of circulating gonadal hormones during puberty and adulthood. This is because gonadal hormones influence the peak and recovery time of the hormonal stress response in males and females, which could cause an overall effect on the stress response (Carey et al., 1995; Handa et al., 1994; McCormick et al., 1998, 2002; Redei et al., 1994; Viau, 2002; Viau & Meaney, 1991; Young et al., 2001). These sex differences in LPS effects may also be attributed in part to the prominent changes in circulating sex steroid hormones, which increase during puberty and influence the immune system. Taken together, there are important age and sex differences that influence the corresponding immune response. The gut microbiome may also play a role in influencing the immune system; however, the mechanism remains uninvestigated.

The Gut Microbiome

The gut microbiome is an important system that has the ability to influence the immune system and inflammatory responses (Rea et al., 2016; Dinan and Cryan, 2013; El Aidy et al., 2014; El Aidy et al., 2015; Moloney et al., 2014; Sampson and Mazmanian, 2015). In more recent years, studies suggest that our gut microbiota can influence central nervous system functioning, which can impact emotional among other kinds of behaviour(Kennedy et al., 2016; Fung et al., 2017; Tillisch et al., 2013; Savignac et al., 2014; Cryan & Dinan, 2012; Bravo et al., 2011). This is due to a bidirectional communication between the brain and the gut (Foster & Neufeld, 2013), commonly referred to as the gut-brain axis (Cryan & Dinan, 2012). It has been theorized that the intestinal bacteria may be a direct contributing factor to our mental health (Schmidt, 2015; Ng et al., 2018), and disruption of the gut-brain axis has been linked with the development of physical and neurological disorders (Ng et al., 2018; Kennedy et al., 2016; Bailey & Cryan, 2017). Studies conducted in germ-free (GF) mice lacking gut microbiota have provided support for the link between microbiota, brain chemistry, and mental health. Compared to normal mice, GF mice display a hyper-reactive HPA axis (Sudo et al., 2004) and increased anxiety-like (Heijtz et al., 2011; Neufeld et al., 2010) and depression-like behaviours (Naseribafrouei et al., 2014; Dinan & Cryan, 2013). However, microbiota colonization decreases anxiety-like behaviour and improves motor activity in GF mice (Heijtz et al., 2011). Overall, gut microorganisms strongly influence the immune system and CNS functioning. One emerging potential therapeutic agent for stress-related GI problems is probiotics.

Probiotics

Probiotics are living microorganisms that can be found in dietary supplements and food products and when ingested in sufficient amounts, provide health benefits to the host (Joint Food and Agriculture Organization/World Health Organization, 2001; Foster & Neufeld, 2013). The immune system can be influenced by probiotics resulting in limiting the production of pro-inflammatory cytokine and inflammation, which can therefore affect the endocrine and nervous systems (Desbonnet et al., 2008; Desbonnet et al., 2010). Thus, probiotics have anti-inflammatory and immune-regulatory properties and are suggested to also improve brain health through the mediation of the immune response (Kennedy et al., 2016). Recent research has also found that probiotics may influence the gut microbiota in neurological and psychiatric disorders (Fung et al., 2017). In naïve rats, the administration of the probiotic *Bifidobacteria infantis* results in a decrease in proinflammatory cytokines (IFN-γ, TNF-α, IL-6) in the blood in response to the forced swim test, a behaviour test used to induce a stress response and examine depression-like behavior (Desbonnet et al., 2008). Another study administered *L. plantarum* PS128 for 28 days to ELS mice and naïve mice. The researchers found that the probiotic decreased pro-inflammatory cytokines (TNF-α, IL-6) and increased anti-inflammatory cytokine (IL-10). In terms of CNS function, locomotor activity and anxiety were tested by open field test and depression was tested by the forced-swim test. Treatment of the probiotic resulted in an increase in locomotor activity, a decrease in anxiety-like behaviour in naïve mice, and a decrease in depression-like behaviour in ELS mice as well (Liu et al., 2016).

The objective of this study is to examine sex differences in the response to probiotic treatment during puberty on LPS-induced immune response by examining sickness behaviour and concentrations of pro-inflammatory cytokines in three different brain regions. LPS is expected to induce a strong immune response, which will be examined by monitoring sickness behaviour and measuring cytokine concentration and expression in pubertal male and female mice. Probiotics in this study are expected to mitigate LPS-induced immune response. Therefore, we hypothesized that mice exposed to probiotics would display less sickness behaviour and cytokine expression, in both mice that were given the immune compromising LPS and those given a control saline solution. Given that previous studies have found that there is a sex difference in response to an immune challenge, we hypothesized that male mice would display greater sickness behaviour, cytokine concentration, and expression compared to females following LPS treatment.

Materials and Methods

Animals

Forty male and forty female CD1 mice were obtained from Charles River Laboratories (St-Constant, Quebec) at three weeks of age. The mice were housed in pairs in polycarbonate Lexan cages (dimensions of 17 x 28 x 12 cm). Mice had *ad libitum* access to food, kefir or control skim milk. Water was not available during the probiotic or control treatment. Feeding bottles were weighed daily to record liquid consumption. There was no difference between kefir and control skim milk intake. Male and female mice were housed in separate rooms; that were maintained on a 14 h light/ 10 h dark cycle (lights off at 10:00am), a constant temperature of 24°C (±2 °C), and 45% humidity. A gradual induction of dusk and dawn was established over 1 h. The Animal Care Committee of the University of Ottawa approved all experimental procedures.

Probiotic treatment

Powdered kefir culture (provided by Lyo San Inc., Lachute, QC) with a lactic acid bacteria concentration of $3.0x10^9$ CFU/g was stored at -20°C. The probiotic kefir was prepared in accordance with Lyo San Inc, by mixing 5g of dry kefir culture in 1L of skim milk. The mixture was kept in an airtight container to inoculate at room temperature 23°C (±2 °C) for 24 hrs prior to being refrigerated at 4°C for a minimum of 8 hrs to end the reaction. A new batch of kefir mixture was prepared three times per week. Every 24 hrs, the treatment bottles were weighed and replaced with pre-weighed bottles. The feeding bottles with kefir mixture were vortexed twice a day in order to prevent clumping and maintain a liquid consistency. Additionally, the feeding bottles for the control group were also checked to maintain consistency. Forty mice (20 males:20 females) received the probiotic kefir and forty (20 males: 20 females) mice received skim milk as a control treatment.

Lipopolysaccharide (LPS) treatment

LPS (from Escherichia coli serotype O26:B6; No. L3755; Sigma- Aldrich Canada, Oakville, ON) was diluted in sterile saline (0.2 mg/ml). LPS was injected intraperitoneally at a dose of 1.5 mg/kg at 6 weeks of age. This dose of LPS treatment has been found to cause mild sickness that only lasts up to 48 hrs (Cai et al., 2016; Girard-Joyal et al., 2015; Ismail & Blaustein, 2013).

Treatments	Males (N=20)	Females (N=20)
Kefir		
LPS	10	10
Saline	10	10
Milk		
LPS	10	10
Saline	10	10

Groups (N=40)

Table 1. Experimental groups.

Sickness monitoring

Sickness behaviour was examined by observing the occurrence of four symptoms; huddling, piloerection, ptosis, and lethargy, as previous studies have found these symptoms to be indicative of sickness as previously described by Kolmogorova et al., (2017) at 30 min, 4 and 8 hrs following LPS or sterile saline treatment in our mice. Two observers, who were blind to treatment conditions, assessed the mice independently using a non-invasive and unbiased approach, as described in Kolmogorova et al., (2017). Each observer assigned a mouse with a sickness score ranging from 0 (no symptoms) to 4 (all four sickness behaviours observed). Sickness checks concluded at 8 hrs, when mice were euthanized.

Euthanasia and tissue collection

Mice were euthanized at 8 hrs following saline or LPS treatment with an intraperitoneal injection of Euthanyl (pentobarbital) (prepared from Euthansol; Merck Animal Intervet Canada Corp; Kirckland, Quebec). Brains were extracted and frozen using liquid nitrogen and stored in aluminium foil in -80°C for further cytokine analysis. Brain samples were later sliced and dissected to collect the prefrontal cortex (PFC), the hypothalamus and the hippocampus following the schematics from *The Mouse Brain Atlas in Stereotaxic Coordinates* (Franklin & Paxinos, 1997).

Real-time qPCR

Messenger RNA (mRNA) was extracted from fresh frozen brain tissue using Isol-RNA lysis Reagent (Cat. No. 2302700, Fisher Scientific). Extracted RNA was exposed to DNAse to remove any genomic DNA prior to cDNA synthesis using the QuantiTect Reverse Transcription kit (Cat. No. 205311, Qiagen). cDNA aliquots were obtained from the extraction to be used in the following qPCR reactions. Relative gene expression was measured using the RealMasterMix Fast SYBR kit (Cat. No. 1725201, Bio-Rad) in 10 µL reactions on a CFX96TOUCH real time PCR machine. All primers were ordered through Integrated DNA Technologies. The efficiency of the primers was determined using the slope of the relation between RNA quantity and cycle thresholds (CT) using Bio-Rad software. All primer pairs in this experiment achieved reaction efficiencies between 90% and 110%. All primers were diluted to a final concentration of 0.3 µM for the real-time PCR reaction. The sequences for the primers were as follows:

Target Gene	Forward	Reverse
β-actin	GAACCCTAAG GCCAACCGTG	GGTACGACCAG AGGCATACAGG
IL-1β	TCTTGGGACT GATGCTGGTG	CAGAATTGCCAT TGCACA ACTC
TNFα	GCCTATGTCTC AGCCTCTTCTC	GCCATTTGGGA ACTTCTCATCC
IL-6	GCCTTCTTGG GACTGATGCT	GCCATTGCACA ACTCTTTTCTC

Table 2. Summary of Primer sequences.

β-actin is the housekeeping gene and was not significantly different amongst experimental groups; therefore, it was used as a reference for all samples. For each reaction, the quantitative threshold amplification cycle number (Cq) was determined, and the 2−ΔΔCq method was used to calculate the relative gene expression of each gene in question.

Statistical analysis

Sickness behaviour and cytokine measures were imported into IBM SPSS Statistics (version 22) for three-way analysis of variance (ANOVA) to examine the effects of sex (males or females), treatment (saline or LPS) and probiotic (kefir or milk). This was followed by pairwise comparisons using the Bonferroni correction, when appropriate. For all tests, the criterion for statistical significance was set to $p < 0.05$.

Results

Sickness behavior

LPS treatment induced sickness behavior in all mice (Fig. 1 and 2). Three-way mixed ANOVA revealed main effects of sex ($F_{(1,65)}=17.59$, $p < 0.01$, $\eta_p^2= 0.213$), LPS treatment ($F_{(1,65)}=2778.713$, $p < 0.01$, $\eta_p^2= 0.977$), and a sex × LPS treatment interaction ($F_{(1,65)}=15.57$, $p < 0.01$, $\eta_p^2= 0.193$). Pairwise comparisons revealed that all LPS-treated males displayed more sickness behavior than female counterparts at 30 min (mean difference; MD = 0.938, standard error; SE = 0.307, $p = 0.03$; MD=1.132, SE = 0.307, $p < 0.01$, respectively) and 4 h (MD = 0.472, SE = 0.141, $p < 0.05$; MD = 0.333, SE = 0.141, $p = 0.021$, respectively), regardless of probiotic treatment. LPS-treated males exposed to kefir showed more sickness behaviour at 2 hrs (MD = 1.063, SE = 0.273, $p < 0.01$), and 6 hrs (MD = 0.549, SE = 0.119, $p < 0.01$) compared to their female counterparts. LPS-treated females exposed to kefir showed significantly more sickness symptoms at 30 min (MD = 0.688, SE = 0.307, $p = 0.028$) but less symptoms at 6 hrs (MD = 0.486, SE = 0.119, $p < 0.01$) compared to LPS-treated females exposed to milk control condition.

Figure 1. Mean (±SEM) sickness score in 6-week-old male (n = 40) mice treated with saline or LPS and exposed to probiotics or milk control.

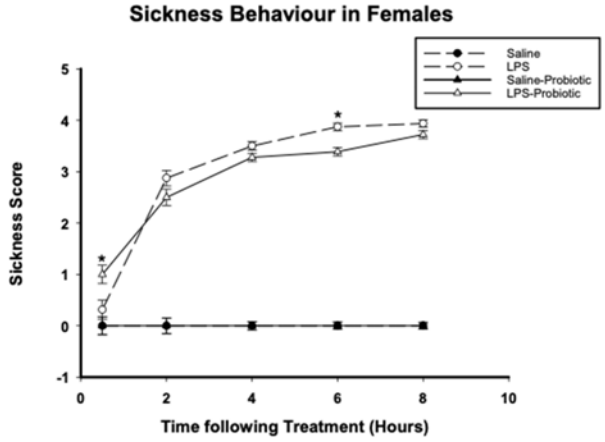

Figure 2. Mean (±SEM) sickness score in 6-week-old female (n = 40) mice treated with saline or LPS and exposed to probiotic or milk control. The asterisks (*) denote significant treatment differences between probiotics and the milk control. ($p < 0.05$) at specified time points.

Pro- Inflammatory Cytokines mRNA Expression in the Hypothalamus, Hippocampus and Prefrontal Cortex Following LPS Treatment

Interleukin-1 Beta (IL-1β) expression. In the hypothalamus, three-way ANOVA revealed main effects of sex ($F_{(1,34)}$=5.281, p = 0.028, η_p^2= 0.134) and LPS treatment ($F_{(1,34)}$=13.62, $p < 0.05$, η_p^2= 0.286) and a significant sex × LPS treatment interaction ($F_{(1,34)}$=5.35, p =0.027, η_p^2= 0.136) on IL-1β mRNA expression. Pairwise comparisons revealed that LPS-treated females displayed more IL-1β mRNA expression compared to their male counterparts in both the milk control (mean difference; MD = 15.726, standard error; SE = 6.863, p = 0.020) and kefir (MD = 15.726, SE = 6.863, p = 0.028) conditions. Additionally, LPS-treated females showed more IL-1β mRNA expression in the hypothalamus compared to saline controls, in both the kefir (MD = 22.97, SE = 6.571, $p <$ 0.05) and milk (MD = 17.36, SE = 6.863, p = 0.016) conditions.

A three-way ANOVA also found a main effect of LPS treatment ($F_{(1,35)}$=10.17, p =0.003, η_p^2= 0.225) on IL-1β mRNA expression in the hippocampus. Pairwise comparison showed that LPS-treated females display greater IL-1β mRNA expression than saline-treated counterparts, regardless of kefir (MD=36.13, SE=12.04, p = 0.005) and milk (MD = 27.39, SE = 12.63, p = 0.037) treatment.

In the prefrontal cortex (PFC), a three-way ANOVA displayed a main effect of LPS treatment ($F_{(1,35)}$=17.54, $p < 0.01$, η_p^2= 0.334) and a significant sex × LPS treatment interaction ($F_{(1,35)}$=9.25, p =0.004, η_p^2= 0.209) in IL-1β mRNA expression. Pairwise comparison showed that within the PFC LPS-treated females exposed to the milk control showed more IL-1β mRNA expression compared to their male counterparts (MD = 34.22, SE = 7.51, $p < 0.01$). This sex difference is absent in mice exposed to the kefir. LPS-injected females that were exposed to kefir showed less IL-1β mRNA expression (MD = 23.22, SE = 7.51, p =0.004) compared to milk controls. LPS-treated females showed more IL-1β mRNA expression compared to saline-treated controls (MD = 37.87, SE = 7.51, $p < 0.01$) in the milk condition only. This treatment difference is absent in mice exposed to kefir.

Tumor necrosis factor alpha (TNFα) expression. Within the hypothalamus, three-way ANOVA revealed main effects of sex ($F_{(1,38)}$ = 8.339, p = 0.006, η_p^2 = 0.180) and LPS treatment ($F_{(1,38)}$ = 25.74, $p < 0.01$, η_p^2 = 0.404) and a significant sex × LPS treatment interaction ($F_{(1,38)}$ = 6.218, p = 0.017, η_p^2 = 0.141) on TNFα mRNA expression. Pairwise

comparisons showed that LPS-treated females exposed to kefir displayed greater TNFα mRNA expression compared to their male counterparts (MD = 5.188, SE = 1.442, p = 0.01). In mice exposed to the milk control, LPS-treated males show more TNFα mRNA expression compared to saline-treated controls (MD = 2.930, SE = 1.374, p = 0.040). This treatment difference is absent in mice exposed to kefir. In both the kefir (MD = 5.951, SE = 1.442, p < 0.01) and milk (MD = 4.706, SE = 1.442, p = 0.002) conditions LPS-treated females show more TNFα mRNA expression compared to saline treated controls.

A three-way ANOVA also found a main effect of LPS treatment ($F_{(1,36)}$=12.37, p < 0.05, η_p^2= 0.256) and a significant probiotic treatment × LPS treatment interaction in TNFα mRNA expression in the hippocampus ($F_{(1,34)}$=6.467, p =0.015, η_p^2= 0.152). Pairwise comparison showed that LPS-treated females exposed to kefir show less TNFα mRNA expression (MD = 2.396, SE = 0.941, p = 0.015) compared to milk controls. Both LPS-treated females and males exposed to milk control displayed more TNFα mRNA expression compared to saline-treated counterparts (MD = 3.317, SE = 0.901, p < 0.05; MD = 2.143, SE = 0.859, p = 0.017, respectively). This effect of LPS treatment on TNFα mRNA expression in the hippocampus was absent in males and females exposed to kefir.

Within the PFC, a three-way ANOVA found main effects of sex ($F_{(1,34)}$=5.913, p =0.020, η_p^2= 0.148) and LPS treatment ($F_{(1,34)}$=11.639, p =0.002, η_p^2= 0.255) and a significant sex × LPS treatment interaction ($F_{(1,34)}$=7.028, p = 0.012, η_p^2= 0.171) for TNFα mRNA expression. Pairwise comparisons showed that LPS-treated females exposed to the milk control display more TNFα mRNA expression in the PFC compared to their male counterparts (MD = 13.580, SE = 4.011, p = 0.002). Moreover, LPS-treated females exposed to milk control also showed more TNFα mRNA expression in the PFC compared to saline-treated counterparts (MD = 16.684, SE = 4.011, p < 0.01). However, LPS-treated females exposed to kefir show less TNFα mRNA expression in the PFC compared to counterparts exposed to milk control (MD = 9.546, SE = 4.011, p = 0.023).

Interleukin-6 (IL-6) expression. In the hypothalamus, three-way ANOVA revealed a main effect of LPS treatment on IL-6 mRNA expression in the hypothalamus ($F_{(1,38)}$=16.92, p < 0.01, η_p^2= 0.308). Pairwise comparisons revealed that, in both kefir (MD = 6.334, SE = 2.73, p = 0.026) and milk conditions (MD = 8.733, SE = 2.61, p = 0.002) LPS-treated females showed more IL-6 mRNA expression compared to their saline-treated counterparts. LPS- treated males in the milk condition show more IL-6 mRNA expression (MD = 5.26, SE = 2.605, p = 0.051) to saline controls. This LPS-induced increase in IL-6 mRNA cytokine expression was eliminated with probiotic treatment. A three-way ANOVA also found a main effect of LPS treatment on IL-6 mRNA expression ($F_{(1,34)}$=15.49, p < 0.01, η_p^2= 0.313), in the hippocampus. Pairwise comparison revealed that in mice treated with kefir, LPS-injected females showed more IL-6 mRNA expression compared to their male counterparts (MD = 7.615, SE = 3.21, p = 0.023). Regardless of the probiotic treatment, LPS-treated females in the kefir (MD = 7.744, SE = 3.21, p = 0.021) and milk (MD = 8.859, SE = 3.21, p = 0.009) conditions showed more IL-6 mRNA expression compared to saline-injected controls. LPS-treated males in the milk conditions showed more IL-6 mRNA cytokine expression (MD = 7.744, SE = 3.208, p = 0.027) compared to saline-injected controls. Again, this LPS-induced increase in IL-6 mRNA cytokine expression was eliminated with probiotic treatment in the hippocampus of male mice.

Within the prefrontal cortex, three-way ANOVA found a main effect of LPS treatment on IL-6 mRNA expression ($F_{(1,31)}$=16.41, p < 0.01, η_p^2= 0.346). In mice treated with probiotics, LPS-injected females showed greater IL-6 mRNA expression compared to their male counterparts (MD = 7.908, SE = 3.787, p = 0.045). Pairwise comparison showed that regardless of the probiotic treatment, when treated with LPS females in both the kefir (MD = 13.94, SE = 3.79, p < 0.05) and milk (MD = 9.19, SE = 4.02, p = 0.029) conditions show more IL-6 compared to saline-injected controls.

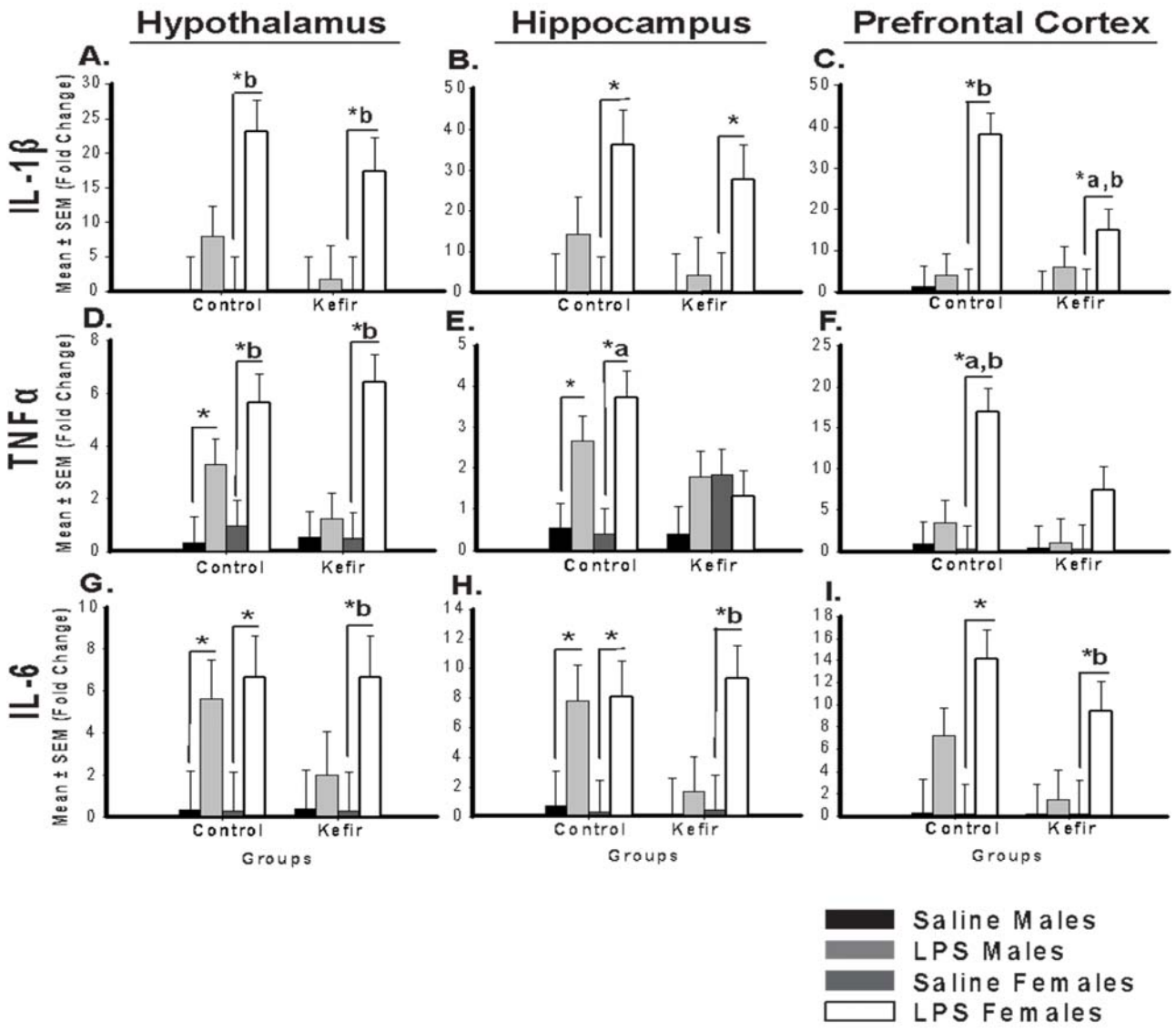

Figure 3. Mean (± SEM) fold change of IL-1β (A, B, C) TNFα (D, E, F) and IL-6 (G, H, I) mRNA expression in the hypothalamus, hippocampus and prefrontal cortex in 6-week-old male and female mice, 8 h following LPS treatment. The asterisk (*) denotes a significant difference between saline or LPS treatment conditions ($p < 0.05$). The (a) denotes a significant difference of probiotic treatment within the same sex. The (b) denotes a significant difference of LPS treatment between sexes.

Discussion

The gut microbiome exerts a strong influence on the immune system. However, the effect of probiotics on the immune response during puberty, a vulnerable period in development, was unknown. The current study examined sex-specific responses to LPS and the possible mitigating properties of probiotic treatment on the immune response. Here, we observed that exposure to LPS during puberty induced an increase in sickness behaviour and pro-inflammatory mRNA expression within the hypothalamus, hippocampus and the prefrontal cortex of both male and female mice. Similar to previous findings (Girard-Joyal et al., 2015; Cai et al., 2016), males displayed greater sickness behaviour compared to their female counterparts in a time-specific manner. Conversely, females displayed greater central mRNA expression of pro-inflammatory cytokines. Probiotic treatment lessened the sickness behavior in a time specific manner

and pro-inflammatory cytokine mRNA expression in a region-specific manner.

Exposure to an immune challenge such as LPS causes a robust immune response that is seen at both physiological and behavioral levels (Bilbo & Schwarz, 2009; Kentner & Pittman, 2010). The severity of LPS-induced sickness behaviours varies over time following infection in a sex-specific manner. Males tend to show greater and prolonged sickness behaviours (Girard-Joyal et al., 2015; Cai et al., 2016). Our results are consistent with previously published work (Cai et al., 2016; Sharma et al., 2018; Murray et al., 2019) and demonstrate that LPS induces greater sickness response in pubertal male mice in comparison to their female counterparts. Male mice showed more sickness at 30 min and at 4 hrs following LPS injection compared to their female counterparts. This sex difference is likely due to gonadal steroid hormones (Foo et al., 2016; Chrousos, 2010; Pittman, 2011). According to Cai et al. (2016), gonadectomized mice displayed significantly more sickness symptoms compared to their sham-operated counterparts 24 hrs following treatment, suggesting that gonadal hormones play a role in decreasing the severity of sickness behavior. Testosterone is a known immune suppressor (Wichmann et al., 1997; Kane & Ismail, 2017; Foo et al., 2016; Alexander and Stimson, 1988; Cutolo et al., 1996; Danel et al., 1983; Roberts et al., 2001; Wunderlich et al., 2002), while estradiol varies in its function. Estradiol can function as an immune suppressor (Kane & Ismail, 2017; Foo et al., 2016; Schuurs & Verheul, 1990; Razmara et al., 2007) or as an immune enhancer by mediating cytokine levels (Grimaldi et al., 2005; Orbach & Shoenfeld, 2007). Females are behaviorally and potentially immunologically protected from some types of inflammatory disease, which is thought to be due to the anti-inflammatory properties of estradiol and progesterone (Bekhbat & Neigh, 2018; Czlonkowska et al., 2006). Both testosterone and estradiol suppress inflammation at the physiological level, and our results indicate that these hormones also have the potential to impact stress-induced inflammation in the brain.

Pro-inflammatory cytokines, like IL-1β, TNFα and IL-6, are known to promote inflammation (Vilcek, 1998; Bluthé et al., 1999; Leon et al., 1999; Sharma et al., 2018; Bekhbat & Neigh, 2018; Tonelli et al., 2008). We hypothesized that cytokine mRNA expression would differ depending on sex and exposure to probiotics. More specifically, due to the increased sickness behavior displayed by male mice, we predicted that males would also display greater inflammation within the brain in response to LPS treatment. In the current study, LPS treatment caused a significant increase in pro-inflammatory cytokines, IL-1β, TNFα and IL-6 mRNA expression, in the hypothalamus, hippocampus and prefrontal cortex in both males and females, compared to saline controls. Contrary to our predictions, females displayed greater central cytokine mRNA expression, which may allude to enduring LPS-induced depression-like behaviour (Murray et al., 2019). Specifically, pubertal females that were treated with LPS showed greater IL-1β mRNA expression in the hippocampus and greater IL-6 mRNA expression in the prefrontal cortex. However, our findings are consistent with previously published work showing that women experience stronger pro-inflammatory responses during infection and are also at a greater risk to developing depression and anxiety disorders compared to men (Engler et al., 2016). Moreover, women tend to react with a stronger inflammatory and innate immune response to infections (Engler et al., 2016; Furman et al., 2014; Klein et al., 2010; Marriott & Huet-Hudson, 2006; Villacres et al., 2004; Verthelyi, 2001; Weinstein et al., 1984). Taken together, the robust central cytokine response in pubertal females alludes to an increased sensitivity to stressors, which could result in an enduring detrimental effect on mental health.

Due to evidence suggesting the gut microbiome influences stress and inflammation (Cryan & Dinan, 2012; Dinan & Cryan, 2013; El Aidy et al., 2015; El Aidy et al., 2014; Moloney et al., 2014; Sampson & Mazmanian, 2015), we hypothesized that mice treated with probiotics would show reduced sickness behavior and cytokine mRNA expression, in both sexes. LPS-injected female mice treated with probiotics showed significantly more sickness symptoms at 30 minutes but less symptoms at 6 hrs compared to LPS-treated females not

exposed to probiotics. These findings are consistent with published work (Bouman et al., 2005; Darnall & Suarez, 2009) and show that females tend to have a more vigorous initial cellular and humoral immune reaction and recover quicker from infections compared to their male counterparts. It is arguable that female mice further benefited from probiotics causing an earlier onset of sickness symptoms as an adaptive behavioural response, which enhanced their adaptive psychophysiological mechanism (Dhabhar, 2014) to overcome sickness.

Intestinal bacteria as well as probiotics have immunomodulatory properties (Desbonnet et al., 2008; Desbonnet et al., 2010). In the current study, male mice displayed increased expression of TNFα and IL-6 in the hypothalamus and hippocampus; however, this effect was reduced with probiotic treatment. Overall, males had a reduced central cytokine response compared to females and probiotic treatment led to a cytokine reduction in both males and females, eliminating sex differences. Probiotic treatment also caused a reduction in LPS-induced inflammation in female mice. Specifically, in the hippocampus TNFα and IL-6 were reduced following probiotic treatment in females, which suggests that beneficial microbes obtained from probiotics mediate the inflammatory response. Our findings indicate that treatment with probiotics preceding an immune challenge decreases the immune response at 8 hours post-infection. These findings are consistent with other published results. A study conducted in rats found similar beneficial effects of probiotics on inflammation; elevated pro-inflammatory cytokines induced by maternal separation were restored to normal levels after subsequent treatment with the probiotic *Bifidobacterium infantis* (Desbonnet et al., 2010). A human study conducted on patients of major depressive disorder found that following probiotic treatment, TNFα and IL-6 both decreased in concentration (Dowlati et al., 2010). This effect can be explained by probiotics having a mitigating effect on the HPA axis (Bravo et al., 2011; Gareau et al., 2011). Taken together, probiotic treatment reduces pro-inflammatory responses in both sexes in a cytokine- and region-specific manner.

Our findings in the current study were consistent with previously conducted research. Specifically, male mice display greater sickness behaviour compared to their female counterparts. Female mice display greater central mRNA expression of pro-inflammatory cytokines, and an immune challenge following probiotic treatment results in a decrease in the immune response. Despite these consistencies the study was not without its limitations. We were primarily concerned with the central cytokine response and did not explore the possibility of other peripheral effects. Studies have shown that peripheral cytokines also play a role in LPS-immune response and can impact mental health (Cai et al., 2016; Sharma et al., 2018). Moreover, we did not examine LPS-induced damage to the gut, which may have had an impact on the immune response. The current study only examined pubertal mice, as puberty is a critical period in development, however there are age-related differences in response to LPS-treatment (Girard-Joyal et al., 2015; Cai et al., 2016), therefore it would be beneficial in a future study to look at pubertal and adult mice in tandem to determine the effects probiotic treatment has on both sex and age.

It has already been established that the gut plays an important role in responding to an acute sickness. Since the CNS is connected to the gut via the vagus nerve, this nerve becomes of special importance too (Forsythe et al., 2010). Bravo et al. (2011) treated healthy mice with a probiotic, *L. rhamnosus*, to examine the effects probiotics had on anxiety- and depressive-like behavior. The results showed that the probiotic did have an alleviating effect, but only when the vagus nerve was intact. Therefore, it would be an interesting future study to further investigate the role that the vagus nerve plays on the immune response.

Finally, the probiotic that was analyzed in the current study was kefir, which contains a mixture of bacteria (Rosa et al. 2017). In order to identify the mechanism through which kefir mitigates LPS-induced inflammation, future studies should examine specific strains of bacteria, such as *lactobacillus* and determine the role that specific strains have on the immune response.

Conclusion

Pubertal exposure to LPS results in enduring negative programming consequences on the developing brain (Ismail et al., 2011; Laroche et al., 2009), but the mechanism underlying these effects remains unknown. The current study elucidated the impact of the gut microbiome on acute immune responses and gave insight into enduring sex-specific alterations in behavior. These findings further advance our understanding of the mechanism underlying sex-specific pubertal immune responses that are influenced by the gut microbiota. Our results also show that pubertal probiotic treatment can mitigate LPS-induced inflammation within the brain. Research on the effects the gut microbiome on the brain and behaviour is relatively new and our current study provides insight on the modulating effects of the gut microbiome on the immune system. The decrease in LPS-induced inflammation following probiotic treatment is likely protective against enduring alterations on behaviour. Additionally, the sex-specific responses to the immune challenge highlight the importance of considering sex in neuroimmunological studies. This study also encourages future research in the field of probiotics to further investigate the influence of the gut microbiome on the brain, particularly during critical periods in development such as puberty. Taken together, probiotics consumption during puberty could prevent enduring stress-induced negative outcomes on mental health in adulthood such as depression and anxiety.

References

Alexander, J., & Stimson, W. (1988). Sex hormones and the course of parasitic infection. *Parasitology Today*, 4(7), 189-193. doi:10.1016/0169-4758(88)90077-4

Bailey, M. T., & Cryan, J. F. (2017). The microbiome as a key regulator of brain, behavior and immunity: Commentary on the 2017 named series. *Brain, Behavior, and Immunity*, 66, 18-22. doi:10.1016/j.bbi.2017.08.017

Bekhbat, M., & Neigh, G. N. (2018). Sex differences in the neuro-immune consequences of stress: Focus on depression and anxiety. *Brain, Behavior, and Immunity,* 67, 1-12. doi:10.1016/j.bbi.2017.02.006

Bilbo, S. D., & Schwarz, J. M. (2009). Early-life programming of later-life brain and behavior: A critical role for the immune system. *Frontiers in Behavioral Neuroscience,* 3, 14. doi:10.3389/neuro.08.014.2009

Bluthé, R., Castanon, N., Pousset, F., Bristow, A., Ball, C., Lestage, J., Michaud, B., Kelley, W. K., & Dantzer, R. (1999). Central injection of IL-10 antagonizes the behavioural effects of lipopolysaccharide in rats. *Psychoneuroendocrinology*, 24(3), 301-311. doi:10.1016/s0306-4530(98)00077-8

Bouman, A., Heineman, M. J., & Faas, M. M. (2005). Sex hormones and the immune response in humans. *Human Reproduction Update,* 11(4), 411-423. doi:10.1093/humupd/dmi008

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., Bienenstock, J., & Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences*, 108(38), 16050-16055. doi:10.1073/pnas.1102999108

Cai, K. C., Mil, S. V., Murray, E., Mallet, J., Matar, C., & Ismail, N. (2016). Age and sex differences in immune response following LPS treatment in mice. *Brain, Behavior, and Immunity*, 58, 327-337. doi:10.1016/j.bbi.2016.08.002

Carey, M. P., Deterd, C. H., Koning, J. D., Helmerhorst, F., & Kloet, E. R. (1995). The influence of ovarian steroids on hypothalamic-pituitary-adrenal regulation in the female rat. *Journal of Endocrinology*, 144(2), 311-321. doi:10.1677/joe.0.1440311

Chrousos, G. P. (2010). Stress and Sex Versus Immunity and Inflammation. *Science Signaling,* 3(143). doi:10.1126/scisignal.3143pe36

Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience*, 13(10), 701-712. doi:10.1038/nrn3346

Cutolo, M. (1996). Androgen and estrogen receptors are present in primary cultures of human synovial macrophages. *Journal of Clinical Endocrinology & Metabolism,* 81(2), 820-827. doi:10.1210/jc.81.2.820

Czlonkowska, A., Ciesielska, A., Gromadzka, G., & Kurkowska-Jastrzebska, I. (2006). Gender Differences in Neurological Disease: Role of Estrogens and Cytokines. *Endocrine,* 29(2), 243-256. doi:10.1385/endo:29:2:243

Danel, L., Souweine, G., Monier, J., & Saez, S. (1983). Specific estrogen binding sites in human lymphoid cells and thymic cells. *Journal of Steroid Biochemistry,* 18(5), 559-563. doi:10.1016/0022-4731(83)90131-0

Darnall, B. D., & Suarez, E. C. (2009). Sex and gender in psychoneuroimmunology research: Past, present and future. *Brain, Behavior, and Immunity*, 23(5), 595-604. doi:10.1016/j.bbi.2009.02.019

Desbonnet, L., Garrett, L., Clarke, G., Bienenstock, J., & Dinan, T. G. (2008). The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat. *Journal of Psychiatric Research*, 43(2), 164-174. doi:10.1016/j.jpsychires.2008.03.009

Desbonnet, L., Garrett, L., Clarke, G., Kiely, B., Cryan, J., & Dinan, T. (2010). Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. *Neuroscience*, 170(4), 1179-1188. doi:10.1016/j.neuroscience.2010.08.005

Dhabhar, F. S. (2014). Effects of stress on immune function: The good, the bad, and the beautiful. *Immunologic Research*, 58(2), 193-210. doi:10.1007/s12026-014-8517-0

Dinan, T. G., & Cryan, J. F. (2013). Melancholic microbes: A link between gut microbiota and depression? *Neurogastroenterology & Motility*, 25(9), 713-719. doi:10.1111/nmo.12198

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E. K., & Lanctôt, K. L. (2010). A Meta-Analysis of Cytokines in Major Depression. *Biological Psychiatry*, 67(5), 446-457. doi:10.1016/j.biopsych.2009.09.033

El Aidy, S., Dinan, T. G., & Cryan, J. F. (2014). Immune modulation of the brain-gut-microbe axis. *Frontiers in Microbiology,* 5, 146. doi:10.3389/fmicb.2014.00146

El Aidy, S., Dinan, T. G., & Cryan, J. F. (2015). Gut Microbiota: The Conductor in the Orchestra of Immune–Neuroendocrine Communication. *Clinical Therapeutics*, 37(5), 954-967. doi:10.1016/j.clinthera.2015.03.002

Engler, H., Benson, S., Wegner, A., Spreitzer, I., Schedlowski, M., & Elsenbruch, S. (2016). Men and women differ in inflammatory and neuroendocrine responses to endotoxin but not in the severity of sickness symptoms. *Brain, Behavior, and Immunity,* 52, 18-26. doi:10.1016/j.bbi.2015.08.013

Foo, Y. Z., Nakagawa, S., Rhodes, G., & Simmons, L. W. (2016). The effects of sex hormones on immune function: A meta-analysis. *Biological Reviews*, 92(1), 551-571. doi:10.1111/brv.12243

Foster, J. A., & Neufeld, K. M. (2013). Gut-brain axis: How the microbiome

influences anxiety and depression. *Trends in Neurosciences*, 36(5), 305-312. doi:10.1016/j.tins.2013.01.005

Franklin, K. B., & Paxinos, G. (1997). *The mouse brain: In stereotaxic coordinates*. San Diego: Academic Press.

Forsythe, P., Sudo, N., Dinan, T., Taylor, V. H., & Bienenstock, J. (2010). Mood and gut feelings. *Brain, Behavior, and Immunity,* 24(1), 9-16. doi:10.1016/j.bbi.2009.05.058

Fung, T. C., Olson, C. A., & Hsiao, E. Y. (2017). Interactions between the microbiota, immune and nervous systems in health and disease. *Nature Neuroscience*, 20(2), 145-155. doi:10.1038/nn.4476

Furman, D., Hejblum, B. P., Simon, N., Jojic, V., Dekker, C. L., Thiebaut, R., Tibshirani, R. J., & Davis, M. M. (2014). Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination. *Proceedings of the National Academy of Sciences*, 111(2), 869-874. doi:10.1073/pnas.1321060111

Gareau, M. G., Wine, E., Rodrigues, D. M., Cho, J. H., Whary, M. T., Philpott, D. J., MacQueen, G., & Sherman, P. M. (2011). Bacterial infection causes stress-induced memory dysfunction in mice. *Gut,* 60(3), 307-317. doi:10.1136/gut.2009.202515

Girard-Joyal, O., & Ismail, N. (2017). Effect of LPS treatment on tyrosine hydroxylase expression and Parkinson-like behaviors. *Hormones and Behavior*, 89, 1-12. doi:10.1016/j.yhbeh.2016.12.009

Girard-Joyal, O., Faragher, A., Bradley, K., Kane, L., Hrycyk, L., & Ismail, N. (2015). Age and sex differences in c-Fos expression and serum corticosterone concentration following LPS treatment. *Neuroscience*, 305, 293-301. doi:10.1016/j.neuroscience.2015.06.035

Goldman, L., Winget, C., Hollingshead, G., & Levine, S. (1973). Postweaning Development of Negative Feedback in the Pituitary-Adrenal System of the Rat. *Neuroendocrinology,* 12(3), 199-211. doi:10.1159/000122169

Green, M. R., Barnes, B., & Mccormick, C. M. (2012). Social instability stress in adolescence increases anxiety and reduces social interactions in adulthood in male long-evans rats. *Developmental Psychobiology*, 55(8), 849-859. doi:10.1002/dev.21077

Grimaldi, C. M., Hill, L., Xu, X., Peeva, E., & Diamond, B. (2005). Hormonal modulation of B cell development and repertoire selection. *Molecular Immunology*, 42(7), 811-820. doi:10.1016/j.molimm.2004.05.014

Handa, R. J., Burgess, L. H., Kerr, J. E., & Okeefe, J. A. (1994). Gonadal Steroid Hormone Receptors and Sex Differences in the Hypothalamo-Pituitary-Adrenal Axis. *Hormones and Behavior*, 28(4), 464-476. doi:10.1006/hbeh.1994.1044

Heijtz, R. D., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., Hibberd, M. L., Forssberg, H., & Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences*, 108(7), 3047-3052. doi:10.1073/pnas.1010529108

Holder, M. K., & Blaustein, J. D. (2014). Puberty and adolescence as a time of vulnerability to stressors that alter neurobehavioral processes. *Frontiers in Neuroendocrinology*, 35(1), 89-110. doi:10.1016/j.yfrne.2013.10.004

Ismail, N., & Blaustein, J. D. (2013). Pubertal immune challenge blocks the ability of estradiol to enhance performance on cognitive tasks in adult female mice. *Psychoneuroendocrinology*, 38(

7), 1170-1177. doi:10.1016/j.psyneuen.2012.11.003

Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, Córdoba, Argentina, 1-4 October 2001. (2001). Rome: Food and Agriculture Organization of the United Nations. doi:10.1016/j.mcn.2012.10.002

Kane, L., & Ismail, N. (2017). Puberty as a vulnerable period to the effects of immune challenges: Focus on sex differences. *Behavioural Brain Research*, 320, 374-382. doi:10.1016/j.bbr.2016.11.006

Kennedy, P. J., Murphy, A. B., Cryan, J. F., Ross, P. R., Dinan, T. G., & Stanton, C. (2016). Microbiome in brain function and mental health. *Trends in Food Science & Technology*, 57, 289-301. doi:10.1016/j.tifs.2016.05.001

Kentner, A. C., & Pittman, Q. J. (2010). Minireview: Early-Life Programming by Inflammation of the Neuroendocrine System. *Endocrinology*, 151(10), 4602-4606. doi:10.1210/en.2010-0583

Klein, S. L., Jedlicka, A., & Pekosz, A. (2010). The Xs and Y of immune responses to viral vaccines. *The Lancet Infectious Diseases,* 10(5), 338-349. doi:10.1016/s1473-3099(10)70049-9

Kolmogorova, D., Murray, E., & Ismail, N. (2017). Monitoring Pathogen-Induced Sickness in Mice and Rats. *Current Protocols in Mouse Biology,* 7(2), 65-76. doi:10.1002/cpmo.27

Laroche, J., Gasbarro, L., Herman, J. P., & Blaustein, J. D. (2009). Reduced Behavioral Response to Gonadal Hormones in Mice Shipped during the Peripubertal/Adolescent Period. *Endocrinology*, 150(5), 2351-2358. doi:10.1210/en.2008-1595

Liu, Y., Liu, W., Wu, C., Juan, Y., Wu, Y., Tsai, H., Wang, S., & Tsai, Y. (2016). Psychotropic effects of Lactobacillus plantarum PS128 in early life-stressed and naïve adult mice. *Brain Research,* 1631, 1-12. doi:10.1016/j.brainres.2015.11.018

Leon, L. R., Kozak, W., Rudolph, K., & Kluger, M. J. (1999). An antipyretic role for interleukin-10 in LPS fever in mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 276(1). doi:10.1152/ajpregu.1999.276.1.r81

Levitt, P. (2003). Structural and functional maturation of the developing primate brain. *The Journal of Pediatrics*, 143(4), 35-45. doi:10.1067/s0022-3476(03)00400-1

Mantzoros, C. S. (1997). A Longitudinal Assessment of Hormonal and Physical Alterations during Normal Puberty in Boys. V. Rising Leptin Levels May Signal the Onset of Puberty. *Journal of Clinical Endocrinology & Metabolism,*82(4), 1066-1070. doi:10.1210/jc.82.4.1066

Marriott, I., & Huet-Hudson, Y. M. (2006). Sexual Dimorphism in Innate Immune Responses to Infectious Organisms. *Immunologic Research,* 34(3), 177-192. doi:10.1385/ir:34:3:177

Mccormick, C. M., Furey, B. F., Child, M., Sawyer, M. J., & Donohue, S. M. (1998). Neonatal sex hormones have organizational effects on the hypothalamic-pituitary-adrenal axis of male rats. *Developmental Brain Research,* 105(2), 295-307. doi:10.1016/s0165-3806(97)00155-7

Mccormick, C. M., Linkroum, W., Sallinen, B. J., & Miller, N. W. (2002). Peripheral and Central Sex Steroids Have Differential Effects on the HPA Axis of Male and Female Rats. *Stress,* 5(4), 235-247. doi:10.1080/1025389021000061165

Moloney, R. D., Desbonnet, L., Clarke, G., Dinan, T. G., & Cryan, J. F. (2014). The microbiome: Stress, health and

disease. *Mammalian Genome,* 25(1-2), 49-74. doi:10.1007/s00335-013-9488-5

Murray, E., Sharma, R., Smith, K., Mar, K., Barve, R., Lukasik, M., Pirwani, A.F., Malette-Guyon, E., Lamba, S., Thomas B., N., Sadeghi-Emamchaie, H., Liang, J., François Mallet, J., Matar, C., & Ismail, N. (2019). Probiotic consumption during puberty mitigates LPS-induced immune responses and protects against stress-induced depression- and anxiety-like behaviors in adulthood in a sex-specific manner. Brain, Behavior, and Immunity, 81, 198-212. doi: 10.1016/j.bbi.2019.06.016

Naseribafrouei, A., Hestad, K., Avershina, E., Sekelja, M., Linløkken, A., Wilson, R., & Rudi, K. (2014). Correlation between the human fecal microbiota and depression. *Neurogastroenterology & Motility,*26(8), 1155-1162. doi:10.1111/nmo.12378

Neufeld, K. M., Kang, N., Bienenstock, J., & Foster, J. A. (2010). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology & Motility,* 23(3). doi:10.1111/j.1365-2982.2010.01620.x

Ng, Q. X., Peters, C., Ho, C. Y., Lim, D., & Yeo, W. (2018). A meta-analysis of the use of probiotics to alleviate depressive symptoms. *Journal of Affective Disorders*, 228, 13-19. doi:10.1016/j.jad.2017.11.063

Olesen, K. M., Ismail, N., Merchasin, E. D., & Blaustein, J. D. (2011). Long-term alteration of anxiolytic effects of ovarian hormones in female mice by a peripubertal immune challenge. *Hormones and Behavior,* 60(4), 318-326. doi:10.1016/j.yhbeh.2011.06.005

Orbach, H., & Shoenfeld, Y. (2007). Hyperprolactinemia and autoimmune diseases. *Autoimmunity Reviews,*6(8), 537-542. doi:10.1016/j.autrev.2006.10.005

Paris, A., Kelly, P., & Ramaley, J. A. (1973). Effects of Short-Term Stress Upon Fertility. II. After Puberty. *Fertility and Sterility,* 24(7), 546-552. doi:10.1016/s0015-0282(16)39796-5

Pittman, D. Q. (2011). A Neuro-Endocrine-Immune Symphony. *Journal of Neuroendocrinology,* 23(12), 1296-1297. doi:10.1111/j.1365-2826.2011.02176.x

Queen, A. E., Moerdyk-Schauwecker, M., Mckee, L. M., Leamy, L. J., & Huet, Y. M. (2016). Differential Expression of Inflammatory Cytokines and Stress Genes in Male and Female Mice in Response to a Lipopolysaccharide Challenge. *Plos One*, 11(4), 1-13. doi:10.1371/journal.pone.015228

Rea, K., Dinan, T. G., & Cryan, J. F. (2016). The microbiome: A key regulator of stress and neuroinflammation. *Neurobiology of Stress*, 4, 23-33. doi:10.1016/j.ynstr.2016.03.001

Razmara, A., Duckles, S. P., Krause, D. N., & Procaccio, V. (2007). Estrogen suppresses brain mitochondrial oxidative stress in female and male rats. *Brain Research,* 1176, 71-81. doi:10.1016/j.brainres.2007.08.036

Redei, E., Li, L., Halasz, I., Mcgivern, R. F., & Aird, F. (1994). Fast Glucocorticoid Feedback Inhibition of ACTH Secretion in the Ovariectomized Rat: Effect of Chronic Estrogen and Progesterone. *Neuroendocrinology,* 60(2), 113-123. doi:10.1159/000126741

Roberts, C. W., Walker, W., & Alexander, J. (2001). Sex-Associated Hormones and Immunity to Protozoan Parasites. *Clinical Microbiology Reviews,* 14(3), 476-488. doi:10.1128/cmr.14.3.476-488.2001

Romeo, R. D., Lee, S. J., Chhua, N., Mcpherson, C. R., & Mcewen, B. S. (2004). Testosterone Cannot Activate an Adult-Like Stress Response in Prepubertal Male Rats. *Neuroendocrinology,* 79(3),

125-132. doi:10.1159/000077270

Rosa, D. D., Dias, M. M., Grześkowiak, Ł M., Reis, S. A., Conceição, L. L., & Peluzio, M. D. (2017). Milk kefir: Nutritional, microbiological and health benefits. *Nutrition Research Reviews*, 30(01), 82-96. doi:10.1017/s0954422416000275

Sampson, T., & Mazmanian, S. (2015). Control of Brain Development, Function, and Behavior by the Microbiome. *Cell Host & Microbe,* 17(5), 565-576. doi:10.1016/j.chom.2015.04.011

Savignac, H. M., Kiely, B., Dinan, T. G., & Cryan, J. F. (2014). Bifidobacteriaexert strain-specific effects on stress-related behavior and physiology in BALB/c mice. Neurogastroenterology & Motility, 26(11), 1615-1627. doi:10.1111/nmo.12427

Schmidt, C. (2015). Thinking from the Gut. *Nature,* 518(7540). doi:10.1038/518s13a

Schuurs, A., & Verheul, H. (1990). Effects of gender and sex steroids on the immune response. *Journal of Steroid Biochemistry,* 35(2), 157-172. doi:10.1016/0022-4731(90)90270-3

Sisk, C. L., & Foster, D. L. (2004). The neural basis of puberty and adolescence. *Nature Neuroscience*, 7(10), 1040-1047. doi:10.1038/nn1326

Sharma, R., Rooke, J., Kolmogorova, D., Melanson, B., Mallet, J., Matar, C., Schwarz, J., & Ismail, N. (2018). Sex differences in the peripheral and central immune responses following lipopolysaccharide treatment in pubertal and adult CD-1 mice. *International Journal of Developmental Neuroscience*, 71, 94-104. doi:10.1016/j.ijdevneu.2018.07.012

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X., Kubo, C., & Koga, Y. (2004). Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *The Journal of Physiology,* 558(1), 263-275. doi:10.1113/jphysiol.2004.063388

Tillisch, K., Labus, J., Kilpatrick, L., Jiang, Z., Stains, J., Ebrat, B., Guyonnet, D., Legrain-Raspaud, S., Trotin, B., Naliboff, B., & Mayer, E. A. (2013). Consumption of Fermented Milk Product With Probiotic Modulates Brain Activity. *Gastroenterology*, 144(7), 1394-1401. doi:10.1053/j.gastro.2013.02.043

Tonelli, L. H., Holmes, A., & Postolache, T. T. (2008). Intranasal Immune Challenge Induces Sex-Dependent Depressive-Like Behavior and Cytokine Expression in the Brain. *Neuropsychopharmacology,*33(5), 1038-1048. doi:10.1038/sj.npp.1301488

Vázquez, D. M., & Akil, H. (1993). Pituitary-Adrenal Response to Ether Vapor in the Weanling Animal: Characterization of the Inhibitory Effect of Glucocorticoids on Adrenocorticotropin Secretion. *Pediatric Research,*34(5), 646-653. doi:10.1203/00006450-199311000-00017

Verthelyi, D. (2001). Sex hormones as immunomodulators in health and disease. *International Immunopharmacology,* 1(6), 983-993. doi:10.1016/s1567-5769(01)00044-3

Viau, V. (2002). Functional Cross-Talk Between the Hypothalamic-Pituitary-Gonadal and -Adrenal Axes. *Journal of Neuroendocrinology,* 14(6), 506-513. doi:10.1046/j.1365-2826.2002.00798.x

Viau, V., & Meaney, M. J. (1991). Variations in the Hypothalamic-Pituitary-Adrenal Response to Stress during the Estrous Cycle in the Rat. *Endocrinology,* 129(5), 2503-2511. doi:10.1210/endo-129-5-2503

Vilcek, J. (1998). *The cytokines: an overview*. In: Thomson, A. (Ed.), The Cytokine Handbook. Academic Press, San Diego, pp. 1-20.

Villacres, M. C., Longmate, J., Auge, C., & Diamond, D. J. (2004). Predominant type 1 CMV-Specific memory T-helper response in humans: Evidence for gender differences in cytokine secretion. *Human Immunology,* 65(5), 476-485. doi:10.1016/j.humimm.2004.02.021

Weinstein, Y., Ran, S., & Segal, S. (1984). Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. *The Journal of Immunology*, 132(2), 656-661.

Wichmann, M. W., Ayala, A., & Chaudry, I. H. (1997). Male sex steroids are responsible for depressing macrophage immune function after trauma-hemorrhage. *American Journal of Physiology-Cell Physiology,* 273(4). doi:10.1152/ajpcell.1997.273.4.c1335

Wunderlich, F., Benten, W. M., Lieberherr, M., Guo, Z., Stamm, O., Wrehlke, C., Sekeris, C. E., & Mossmann, H. (2002). Testosterone signaling in T cells and macrophages. *Steroids,* 67(6), 535-538. doi:10.1016/s0039-128x(01)00175-1

Young, E. (2001). Effects of Estrogen Antagonists and Agonists on the ACTH Response to Restraint Stress in Female Rats. *Neuropsychopharmacology,* 25(6), 881-891. doi:10.1016/s0893-133x(01)00301-3