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The effects of antimicrobials and lipopolysaccharide on acute immune responsivity in pubertal male and female CD1 mice

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ABSTRACT

Exposure to stress during critical periods of development—such as puberty—is associated with long-term disruptions in brain function and neuro-immune responsivity. However, the mechanisms underlying the effect of stress on the pubertal neuro-immune response has yet to be elucidated. Therefore, the objective of the current study was to investigate the effect antimicrobial and lipopolysaccharide (LPS) treatments on acute immune responsivity in pubertal male and female mice. Moreover, the potential for probiotic supplementation to mitigate these effects was also examined. 240 male and female CD1 mice were treated with one week of antimicrobial treatment (mixed antimicrobials or water) and probiotic treatment (*L. rhamnosis* R0011 and *L. helveticus* R0052 or *L. helveticus* R0052 and *B. longum* R0175) 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 8 h post-injection. Brain, blood, and intestinal samples were collected. The results indicated that the antimicrobial treatment reduced sickness behaviours, and potentiated LPS-induced plasma cytokine concentrations and pro-inflammatory markers in the pre-frontal cortex (PFC) and hippocampus, in a sex-dependent manner. However, probiotics reduced LPS-induced plasma cytokine concentrations along with hippocampal and PFC pro-inflammatory markers in a sex-dependent manner. *L. rhamnosis* R0011 and *L. helveticus* R0052 treatment also mitigated antimicrobial-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.

1. Introduction

Throughout the lifespan, there are multiple periods of development that are sensitive to stress. Exposure to stress during these critical periods can have short and long-term physiological, neural, and behavioral consequences. One of these critical periods is puberty; defined as a period of maturation of reproductive systems into an adult-like phenotype (reviewed in Ref. [40]. Puberty is also a period of development during which the brain is sensitive to stress exposure and neuroendocrine dysregulation [17]. A single dose of the bacterial endotoxin, lipopolysaccharide (LPS; 1.5 mg/kg), during the pubertal stress-sensitive period (6 weeks of age), in CD-1 mice, suppresses sexual receptivity in females [17], induces cognitive deficits [23], and increases anxiety-like behaviours in males and depression-like behaviours in females, in an enduring manner [29]. Moreover, pubertal LPS treatment decreases estrogen receptor-α (ER-α) and increases c-fos expression in adulthood [13,17]. These results suggest that exposure to an immune challenge during puberty impairs reproductive and non-reproductive behaviours through physiological changes in brain structure and function [20].

Immune response to a pathogen differs across age and sex, due in part to circulating gonadal hormones and differential organization of interacting systems. In pubertal mice, LPS exposure induces a hypo-responsive immune response compared to adults. Ten hours following

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LPS injection, pubertal male and female CD-1 mice display less serum pro-inflammatory cytokines, more serum anti-inflammatory cytokines [5], and less sickness behaviours [38] compared to LPS-treated adults. It has been hypothesized that pubertal hypo-responsiveness to an immune challenge can be attributed to the fact that the immune system and the hypothalamic-pituitary-adrenal (HPA) axis have not fully matured yet [20].

Males and females have distinct immune responses. Overall, estradiol acts to enhance immune cell processes, whereas testosterone suppresses the functionality of immune cells. Moreover, pubertal males display greater sickness behaviours (such as hypothermic responses to LPS) and take longer to recover from a sickness compared to females following LPS treatment [5]. In addition to influencing the peripheral immune system, gonadal hormones are directly correlated with microglial functionality in the central nervous system (CNS). Estradiol is positively correlated with microglial hippocampal expression in female rats [34]. Thus, estradiol is theorized to be protective against both peripheral and central inflammation after an acute immune challenge [34].

The gut microbiome is another factor that influences immune reactivity and inflammation. The gut microbiome is a collection of bacteria that are located along the entire digestive tract, and contains 90% of the total microbes that colonize the human body [16]. The bacteria within the microbiome exist in symbiosis with 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: [10]. The mechanism with which the gut microbiome influences neuro-inflammation remains elusive. One potential pathway is through neuro-modulating hormones and derivatives synthesized by the microbiome [6]. Studies have shown that vancomycin-treated male and female C57BL/6 mice display significant reductions in short-chain fatty acid (SCFA) concentrations in colonic tissue compared to controls [12]. Similar studies have shown that treatments with antimicrobial cocktails deplete the microbiota, reduce mitochondrial gene expression in neurons and microglia, and increase neuronal cell death [28]. 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.

Replenishment of specific bacterial strains with the use of probiotics has been shown to be immunomodulatory and have positive effects on immune responsivity. For example, research with Long-Evans rats has shown that exposure to a western diet along with lifelong treatment with *Bifidobacterium longum* R0175 and *Lactobacillus helveticus* R0052 decreases anxiety-like behaviours and increases the peripheral immune response (i.e., IL1β, IL7, GM-CSF, MCP1) of male rats compared to their female counterparts, following exposure to a predator odor stressor [31]. Moreover, 2-weeks of treatment with *B, longum* R0175 and *L. helveticus* R0052 in C57BL/6J mice significantly reduces visceral pain during colorectal distension [2]. This reduction in visceral pain is accompanied by a reduction in plasma stress hormones (i.e., corticosterone, adrenaline/noradrenaline) and the regulation of glucocorticoid mRNA expression. Other research with Sprague-Dawley rats has shown that maternal separation during early infancy alters the normal developmental timing of pubertal onset and treatment with *Lacticaseibacillus rhamnosus* R0011 and *L. helveticus* R0052 mitigates these effects [7]. As such, probiotic treatment may be a viable option to mitigate the effects of pubertal microbial dysbiosis on immune responsivity.

The literature assessing the effects of stress on neuro-immune signaling during puberty remains unclear. Moreover, previous research examining the impact of antimicrobials on stress and immune responses has primarily used adult male mice. Therefore, the objective of the current study is to determine the impact of pubertal antimicrobial and LPS treatment on acute immune responsivity in male and female mice, and to determine if this effect can be mitigated by probiotics. We hypothesized that pubertal antimicrobial, LPS and probiotic supplementation would alter immune responsivity in male and female mice. More specifically, we hypothesized that (1) antimicrobials would potentiate LPS-induced sickness behaviours, plasma cytokine concentrations, and cytokine mRNA expression in the hippocampus and prefrontal cortex (PFC), (2) probiotic supplementation would reduce the effects of antimicrobials and LPS on intestinal weights, LPS-induced sickness behaviours, plasma cytokine concentrations, and cytokine mRNA expression in the hippocampus and PFC, and (3) antimicrobial-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 and PFC. The results from this experiment will provide greater insight into the sex-dependent effects of antimicrobials and probiotics on immune responsivity during puberty.

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. All cohorts of mice were subjected to the same protocols. 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). Each housing room contained a sentinel to ensure that no external pathogens were affecting the health of our mice. 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. Antimicrobial treatment

At five weeks of age, mice were administered 200 μL of mixed broad-spectrum antimicrobial solution or distilled water through gavage twice daily for seven days. The antimicrobial solution was made fresh daily and contained 15 mg/mL of ampicillin (No. BP1760-5, Fisher Scientific, Geel, Belgium), neomycin (No. 480125 GM, EMD Millipore Corp, MA, USA), streptomycin (NO. BP910-50, Alfa 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 h and 1800 h, respectively. This dosage and treatment regimen have been shown to sufficiently suppress total microbial content [49].

2.3. Probiotic supplementation

At five weeks of age (simultaneous to the antimicrobial treatments), mice were exposed to 1 billion CFU/mL of *Lacticaseibacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 in a 95:5 ratio (Lacidofil®; Lallemand Inc., Montreal, QC, Canada), *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* subspecies longum R0175 in a 90:10 ratio (Cerebiome®; Lallemand Inc., Montreal, QC, Canada), 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 antimicrobial

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 0 (day before treatment regimen) was subtracted from day 7 (last day of treatment) and converted to a percentage.

2.5. Lipopolysaccharide administration

Six-week-old mice received an intraperitoneal (ip) injection (1 week following the start of antimicrobial and probiotic treatments) 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. LPS was chosen as our stressor of interest because it activates the immune system in a sex-dependent manner. Moreover, this dose of LPS was chosen because it has been previously shown to induce sexually dimorphic sickness behaviours for approximately 24–48 h and induce alterations to the composition of the gut microbiome [5,29]. It is likely that our male and female mice are at different stages of puberty during this study, however, 6 weeks of age is a stress-sensitive period during which exposure to LPS has enduring effects in both male and female mice [29]. Moreover, under our housing conditions, CD-1 female mice housed in single sex rooms demonstrate vaginal opening approximately 30 days following birth and have their first estrous cycle 20 days post vaginal-opening (N. Ismail and J.D. Blaustein, unpublished observations). Measurements of preputial separation in male mice are difficult to analyze, however, measurements of scrotum width in six-week-old male mice indicate that the scrotum has not reached adult size (Lamba, Murray & Ismail, unpublished observations). Therefore, this suggest that our six-week-old mice are pubertal mice.

2.6. Sickness monitoring

Sickness monitoring was conducted at 2, 4, 6, and 8 h after injection. Assessment of the progression of sickness behaviours followed a non-invasive and unbiased approach with two raters blind to the experimental conditions (as described in Ref. [22]. 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 h 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 3 h of blood collection, samples were centrifuged at 1000×*g* at 20 °C for 15 min 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 300 μM, and hippocampal and PFC tissue was extracted with 2.0 mm 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 was weighed. Whole intestine weights were recorded across groups and were used as a validated method to assess the efficacy of antimicrobial treatments on intestinal physiology and microbial content [49].

2.10. Real-time quantitative polymerase chain reaction (RTqPCR)

mRNA was extracted from hippocampal and PFC tissue using PureLink RNA Mini Kit (No. 12183020; Thermo-Fisher Scientific) and used according to the manufacturer's instructions. 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 using the SsoAdvanced Universal SYBR Green Supermix (No. 1725274; Bio-Rad) in triplicates of 10 μL reactions on the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad). All primers were ordered from Integrated DNA technologies. Primer efficiency was determined using the slope between RNA quantity and cycle thresholds with CFX Maestro software (Bio-Rad). All primer pairs achieved reaction efficiency between 90 and 110%. The primer sequences are as follows: *β-actin* forward: GAACCCTAAGGCCAACCGTG, reverse: GGTACGACCAGAGGCATACAGG; *IL1β* forward: TCTTGGGACTGATGCTGGTG, reverse: CAGAATTGCCATTGCACAACTC; *TNFα* forward: GCCTATGTCTCAGCCTCTTCTC, reverse: GCCATTTGGGAACTTCTCATCC; *IL6* forward: GTTCCTCTCTGCAAGAGACTTC, reverse: CTCCTCTCCGGACTTGTGAA. 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 interleukin-1 beta (IL1β), interleukin-6 (IL6), interleukin-10 (IL10), interleukin-12 (p70) (IL12), interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) were measured with a multiplex bead-based 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 to monitor the inter-assay variation. Minimum detectable concentration (MinDC) was used to determine the sensitivity of the assay and varied depending on the analyte (IL1β = 5.4 pg/ml, IL6 = 1.1 pg/ml, IL10 = 2.0 pg/ml, IL12 = 4.8 pg/ml, IFNγ = 1.1 pg/ml, and TNFα = 2.3 pg/ml). Cross reactivity between the antibodies for each analyte was negligible. The MAGPIX system was used to measure the final cytokine concentrations. Samples with intra-assay CVs greater than 10% were excluded from the analyses.

2.12. Statistical analyses

All statistical analyses were performed using IBM SPSS v20 software. Cases that exceeded the 1.5 interquartile range in boxplot analyses (probiotic consumption, body weight and sickness behaviour data, intestine weights, rt-qPCR, and multiplex data) were considered statistical outliers and were limited, by winsorization to the next outer-most score within the 1.5 interquartile range [15]. For all measures, 2 × 2 x 3 × 2 ANOVAs were performed for sex (male or female), antimicrobial treatment (AMNS or water), probiotic treatment (*L. rhamnosis* R0011 and *L. helveticus* R0052, *L. helveticus* R0052 and *B. longum* R0175, or placebo), and LPS treatment (LPS or saline). For measures of sickness behaviours and probiotic consumption data (mixed ANOVA),

Greenhouse-Geisser corrections were 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_p^2). Statistical significance was set to $p < 0.05$.

3. Results

3.1. Consumption rates of probiotic supplements

The 2 × 2 x 3 × 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 ($F_{(4.4,504.5)} = 20.15$, $p < 0.05$, $\eta_p^2 = 0.16$), and a significant time x sex ($F_{(4.4504.5)} = 3.02$, $p < 0.05$, $\eta_p^2 = 0.03$) interaction. The ANOVA also revealed a significant between-subjects main effect of antimicrobial ($F_{(1,107)} = 12.37$, $p < 0.05$, $\eta_p^2 = 0.11$). Pairwise comparisons showed that regardless of antimicrobial and probiotic treatment, males drank more than females on days 3 and 5 ($MD = 0.38$, $SE = 0.17$, $p < 0.05$; $MD = 0.70$, $SE = 0.25$, $p < 0.05$, respectively). Moreover, regardless of sex and probiotic treatment, water-treated mice drank significantly more than antimicrobial-treated mice ($MD = 0.47$, $SE = 0.14$, $p < 0.05$; Fig. 1A and C).

3.2. Body weight changes

The 2 × 2 x 3 × 2 ANOVA revealed a significant main effect of sex ($F_{(1,228)} = 14.82$, $p < 0.05$, $\eta_p^2 = 0.06$), where males had significantly more percent body weight change after seven days of antimicrobial treatment than females ($MD = 2.11$, $SE = 0.55$, $p < 0.01$). There was also a significant sex x antimicrobial interaction ($F_{(1,228)} = 6.03$, $p < 0.05$, $\eta_p^2 = 0.03$). Pairwise comparisons showed that antimicrobial-treated males gained significantly less weight compared to their water-treated counterparts ($MD = -2.31$, $SE = 0.78$, $p < 0.05$; Fig. 2A). There was no significant body weight change in the females (Fig. 2B).

3.3. Whole intestinal weights

The 2 × 2 x 3 × 2 ANOVA found significant main effects of sex ($F_{(1,212)} = 178.49$, $p < 0.01$, $\eta_p^2 = 0.46$), LPS ($F_{(1,212)} = 337.76$, $p < 0.01$, $\eta_p^2 = 0.61$), and antimicrobial ($F_{(1,212)} = 479.04$, $p < 0.01$, $\eta_p^2 = 0.71$). As well, significant sex x LPS ($F_{(1,212)} = 8.66$, $p < 0.01$, $\eta_p^2 = 0.04$), LPS x antimicrobial ($F_{(1,212)} = 23.79$, $p < 0.01$, $\eta_p^2 = 0.10$), and LPS x probiotic ($F_{(1,212)} = 4.13$, $p < 0.05$, $\eta_p^2 = 0.04$) interactions were found. Pairwise comparisons showed that antimicrobial-treated mice had significantly higher intestinal weights compared to water-treated counterparts ($MD = 0.86$, $SE = 0.04$, $p < 0.01$). Males had significantly higher intestinal weight after LPS treatment compared to LPS-treated females ($MD = 0.41$, $SE = 0.06$, $p < 0.01$; Fig. 2C and D). As well, antimicrobial-treated mice had significantly higher intestinal weight after LPS, when compared to their water-treated counterparts ($MD = 0.67$, $SE = 0.06$, $p < 0.01$; Fig. 2C and D).

3.4. Sickness behaviours

The 2 × 2 x 3 × 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 ($F_{(2.9,614.3)} = 714.05$, $p < 0.01$, $\eta_p^2 = 0.77$) and significant time x LPS ($F_{(2.9,614.3)} = 727.46$, $p < 0.01$, $\eta_p^2 = 0.77$) and time x sex x LPS ($F_{(2.9,614.3)} = 3.23$, $p < 0.05$, $\eta_p^2 = 0.02$) interactions. The ANOVA also revealed significant main effects of sex ($F_{(1,214)} = 10.78$, $p < 0.01$, $\eta_p^2 = 0.05$) and LPS ($F_{(1,214)} = 2516.31$, $p < 0.001$, $\eta_p^2 = 0.92$), and significant sex x LPS ($F_{(1,214)} = 5.22$, $p < 0.05$, $\eta_p^2 = 0.02$), sex x antimicrobial ($F_{(1,214)} = 7.62$, $p < 0.05$, $\eta_p^2 = 0.03$), LPS x antimicrobial ($F_{(1,214)} = 17.94$, $p < 0.01$, $\eta_p^2 = 0.08$), antimicrobial x probiotic ($F_{(2,214)} = 3.06$, $p = 0.05$, $\eta_p^2 = 0.03$), and sex x LPS x antimicrobial ($F_{(1,214)} = 3.59$, $p < 0.05$, $\eta_p^2 = 0.04$) interactions.

Pairwise comparisons showed that LPS and antimicrobial-treated males displayed significantly less sickness behaviours at 8h following treatment compared to their LPS and water-treated counterparts ($MD = 0.25$, $SE = 0.12$, $p < 0.05$; Fig. 3). Moreover, saline and antimicrobial-treated males displayed significantly more sickness behaviours compared to saline and antimicrobial-treated female counterparts ($MD = 0.49$, $SE = 0.09$, $p < 0.01$; Fig. 3). Interpretations of the antimicrobial x probiotic interaction showed that antimicrobial-treated mice exposed to *L. rhamnosis* R0011 and *L. helveticus* R0052 had significantly less sickness behaviours compared to antimicrobial-treated counterparts exposed to placebo or to *L. helveticus* R0052 and *B. longum* R0175 ($MD = 0.22$, $SE = 0.08$, $p < 0.05$; $MD = 0.21$, $SE = 0.08$, $p < 0.05$, respectively; Fig. 3).

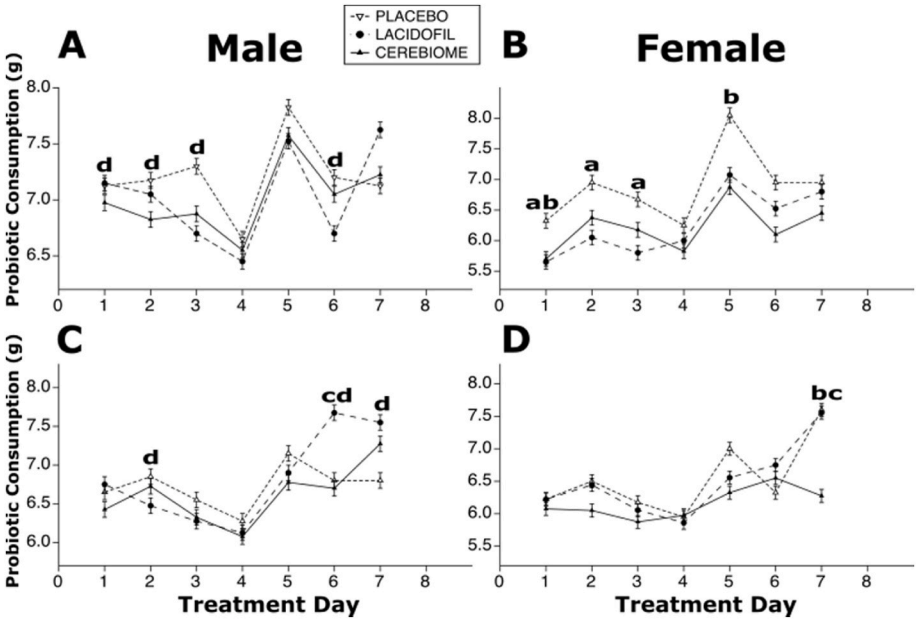

Fig. 1. Daily consumption volume of six-week-old (A) male water-treated mice, (B) male antimicrobial-treated mice, (C) female water-treated mice and (D) female antimicrobial-treated mice supplemented with either placebo, *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®), or *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®). Data represented as mean consumption (±SEM), n = 78–80/group. (a) denotes a significant difference between placebo and *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®) treatments ($p < 0.05$), (b) denotes a significant difference between placebo and *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®) treatments ($p < 0.05$) and (c) denotes a significant difference between *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®) and *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®) treatments ($p < 0.05$), (d) denotes a significant difference between antimicrobial and water-treated mice ($p < 0.05$).

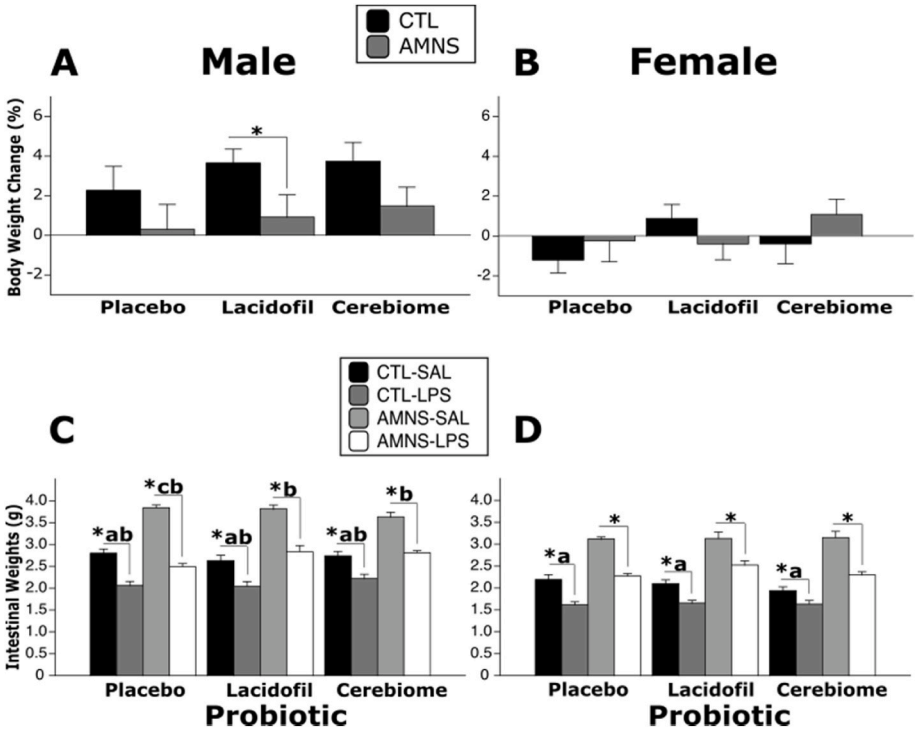

Fig. 2. Percent body weight change in six-week-old (A) male and (B) female mice treated with water (CTL) or antimicrobials (AMNS) and supplemented with either placebo, *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®), or *L. helveticus* R0052 and *B. longum* R0175. Data represented as a mean percentage (±SEM), n = 18–20/group. (*) denotes a significant difference between antimicrobial-treated and water-treated mice ($p < 0.05$). Whole intestinal weights of (C) male and (D) female six-week-old mice treated with saline (SAL) or LPS, water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), and supplemented with either placebo, *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®), or *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®). Data represented as mean (±SEM), n = 8–10/group. (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (a) denotes a significant difference between antimicrobial and water-treated counterparts ($p < 0.05$), (b) denotes a significant difference between male and female counterparts, and (c) denotes a significant difference between placebo and *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®) treatments.

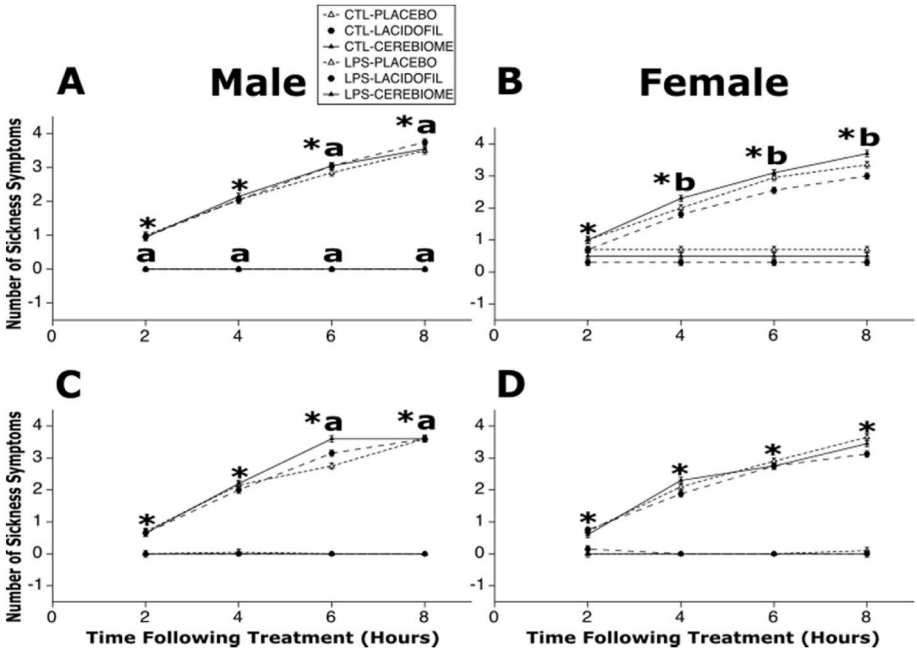

Fig. 3. Acute sickness scores of six-week-old (A) male water-treated mice, (B) male antimicrobial-treated mice, (C) female water-treated mice, and (D) female antimicrobial-treated mice supplemented with either placebo, *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®), or *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®) and treated with saline (SAL-PLACEBO, SAL-LACTO, SAL-CEREBIOME) or LPS (LPS-PLACEBO, LPS-LACTO, LPS-CEREBIOME). Data represented as mean sickness scores (±SEM), n = 28–30/group. (*) denotes a significant difference between saline and LPS-treated mice ($p < 0.05$). (a) denotes a significant difference between antimicrobial and water-treated mice ($p < 0.05$). (b) denotes a significant difference between *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®) and *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®) treatments ($p < 0.05$).

3.5. Assessment of hippocampal IL1β, TNFα and IL6 mRNA cytokine expression with RT-qPCR

The ANOVA found a significant main effect of sex for IL1β ($F_{(1,89)} = 8.02$, $p < 0.01$, $\eta p2 = 0.08$) and IL6 ($F_{(1,89)} = 9.57$, $p < 0.01$, $\eta p2 = 0.10$). A main effect of LPS was found for IL1β ($F_{(1,89)} = 53.50$, $p < 0.01$, $\eta p2 = 0.38$), TNFα ($F_{(1,89)} = 24.66$, $p < 0.01$, $\eta p2 = 0.22$), and IL6 ($F_{(1,89)} = 62.65$, $p < 0.001$, $\eta p2 = 0.41$). A main effect of probiotics was found for IL6 ($F_{(1,89)} = 5.47$, $p < 0.01$, $\eta p2 = 0.11$). There was also a significant sex x LPS interaction for IL1β ($F_{(1,89)} = 6.62$, $p < 0.01$, $\eta p2 = 0.07$) and IL6 ($F_{(1,89)} = 13.65$, $p < 0.01$, $\eta p2 = 0.13$) along with a significant antimicrobial x probiotic ($F_{(2,89)} = 3.27$, $p < 0.01$, $\eta p2 = 0.07$), and LPS x probiotic interactions ($F_{(2,89)} = 4.84$, $p < 0.01$, $\eta p2 = 0.10$) for IL6.

Pairwise comparisons showed that regardless of sex, antimicrobial, and probiotic treatment, LPS-treated mice displayed significantly greater IL1β ($MD = 20.92$, $SE = 2.86$, $p < 0.01$), TNFα ($MD = 5.01$, $SE = 1.01$, $p < 0.01$; Fig. 4C and D) and IL6 ($MD = 10.20$, $SE = 1.29$, $p < 0.01$; Fig. 4E and F) mRNA expression in comparison to their saline-treated counterparts. Moreover, regardless of antimicrobial and probiotic treatment, LPS-treated female mice displayed significantly greater IL1β ($MD = 15.46$, $SE = 4.03$, $p < 0.01$; Fig. 4A and B) and IL6 ($MD = 8.75$, $SE = 1.81$, $p < 0.01$) mRNA expression in comparison to male counterparts.

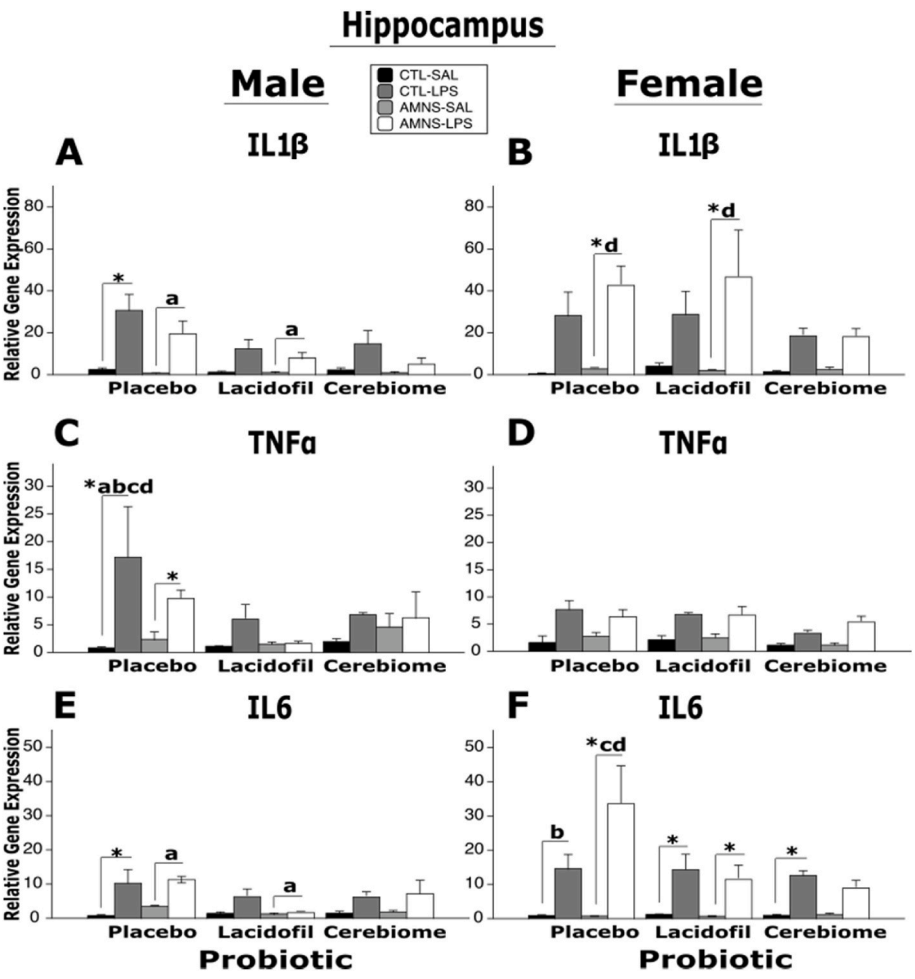

Fig. 4. Acute hippocampal (A) IL1β mRNA expression of males, (B) IL1β mRNA expression of females, (C) TNFα mRNA expression of males, (D) TNFα mRNA expression of female mice, (E) IL6 mRNA expression of males, and (F) IL6 mRNA expression of female six-week-old mice treated with saline (SAL) or LPS, water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), and supplemented with either placebo, *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®), or *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®). Data represented as mean fold change (±SEM), n = 18–20/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (a) denotes a significant difference between male and female counterparts ($p < 0.05$), (b) denotes a significant difference between water and antimicrobial treatments (c) denotes a significant difference from the *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®) experimental condition ($p < 0.05$), and (d) denotes a significant difference from the *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®) experimental condition ($p < 0.05$).

Lastly, regardless of sex, LPS- and antimicrobial-treated mice supplemented with placebo displayed significantly greater IL6 mRNA expression in comparison to LPS and antimicrobial treated mice supplemented with *L. rhamnosis* R0011 and *L. helveticus* R0052.

(*MD* = 15.90, *SE* = 3.14, $p < 0.01$; Fig. 4F) or *L. helveticus* R0052 and *B. longum* R0175 (*MD* = 14.40, *SE* = 3.14, $p < 0.01$; Fig. 4F).

3.6. Assessment of pre-frontal cortex IL1β, TNFα and IL6 mRNA expression with RT-qPCR

The ANOVA revealed a significant main effect of sex for TNFα ($F_{(1,95)} = 24.03$, $p < 0.01$, ηp2 = 0.20) and IL6 ($F_{(1,95)} = 53.32$, $p < 0.01$, ηp2 = 0.36). A main effect of LPS was found for IL1β ($F_{(1,95)} = 46.76$, $p < 0.01$, ηp2 = 0.33), TNFα ($F_{(1,95)} = 26.04$, $p < 0.01$, ηp2 = 0.22), and IL6 ($F_{(1,95)} = 55.57$, $p < 0.01$, ηp2 = 0.37). A main effect of antimicrobial was found for TNFα ($F_{(1,95)} = 7.14$, $p < 0.01$, ηp2 = 0.07) and a main effect of probiotics was found for IL1β ($F_{(2,95)} = 8.62$, $p < 0.01$, ηp2 = 0.15) and TNFα ($F_{(2,95)} = 10.72$, $p < 0.01$, ηp2 = 0.18). A significant sex x LPS interaction was found for TNFα ($F_{(1,95)} = 8.77$, $p < 0.01$, ηp2 = 0.08) and IL6 ($F_{(1,95)} = 30.60$, $p < 0.01$, ηp2 = 0.25) along with a significant LPS x probiotic interaction for IL1β ($F_{(2,95)} = 3.20$, $p < 0.01$, ηp2 = 0.06) and TNFα ($F_{(2,95)} = 6.77$, $p < 0.01$, ηp2 = 0.13). There was also significant sex x antimicrobial ($F_{(1,95)} = 4.98$, $p < 0.01$, ηp2 = 0.05), sex x LPS ($F_{(1,95)} = 8.77$, $p < 0.01$, ηp2 = 0.08), sex x probiotic ($F_{(2,95)} = 4.23$, $p < 0.01$, ηp2 = 0.08), antimicrobial x probiotic ($F_{(2,95)} = 8.89$, $p < 0.01$, ηp2 = 0.16) sex x antimicrobial x probiotic ($F_{(2,95)} = 4.86$, $p < 0.01$, ηp2 = 0.09), and antimicrobial x LPS x probiotic interactions ($F_{(2,95)} = 6.39$, $p < 0.01$, ηp2 = 0.12) for TNFα.

Pairwise comparisons showed that regardless of sex, antimicrobial and probiotic treatment, LPS-treated mice displayed significantly greater IL1β (*MD* = 6.17, *SE* = 0.90, $p < 0.01$; Fig. 5A and B), TNFα (*MD* = 3.64, *SE* = 0.71, $p < 0.01$), and IL6 (*MD* = 10.54, *SE* = 1.41, $p < 0.01$) mRNA expression in comparison to their saline-treated counterparts. Regardless of sex and antimicrobial treatment, LPS-treated mice supplemented with placebo displayed significantly greater IL1β mRNA expression in comparison to LPS-treated mice supplemented with *L. rhamnosis* R0011 and *L. helveticus* R0052 (*MD* = 7.14, *SE* = 1.56, $p < 0.01$) or *L. helveticus* R0052 and *B. longum* R0175 (*MD* = 5.03, *SE* = 1.56, $p < 0.01$; Fig. 5A and B). LPS-treated male mice displayed significantly greater IL6 (*MD* = 18.15, *SE* = 2.01, $p < 0.01$; Fig. 5E and F) and TNFα (*MD* = 5.60, *SE* = 1.00, $p < 0.01$) mRNA expression in comparison to their LPS-treated female counterparts. As well, regardless of LPS and probiotic treatment, male mice treated with antimicrobials displayed significantly greater TNFα mRNA expression in comparison to their water-treated counterparts (*MD* = 5.60, *SE* = 1.00, $p < 0.01$; Fig. 5C and D). Lastly, male mice treated with LPS, antimicrobials, and received placebo displayed significantly greater TNFα mRNA expression in comparison to male mice treated with LPS, antimicrobials, andsupplemented with *L. rhamnosis* R0011 and *L. helveticus* R0052 (*MD* = 20.51, *SE* = 2.46, $p < 0.01$; Fig. 5D) or *L. helveticus* R0052 and *B. longum* R0175 (*MD* = 18.99, *SE* = 2.46, $p < 0.01$; Fig. 5D).

3.7. Assessment of peripheral IFNγ, IL1β, IL6, IL10, IL12, and TNFα concentrations with multiplex immunoassays

The ANOVA found a significant main effect of LPS for IFNγ ($F_{(1,89)} = 188.05$, $p < 0.01$, $\eta_p^2 = 0.68$), IL1β ($F_{(1,89)} = 89.61$, $p < 0.01$, $\eta_p^2 = 0.50$), IL6 ($F_{(1,89)} = 85.21$, $p < 0.01$, $\eta_p^2 = 0.49$), IL10 ($F_{(1,89)} = 152.86$, $p <$

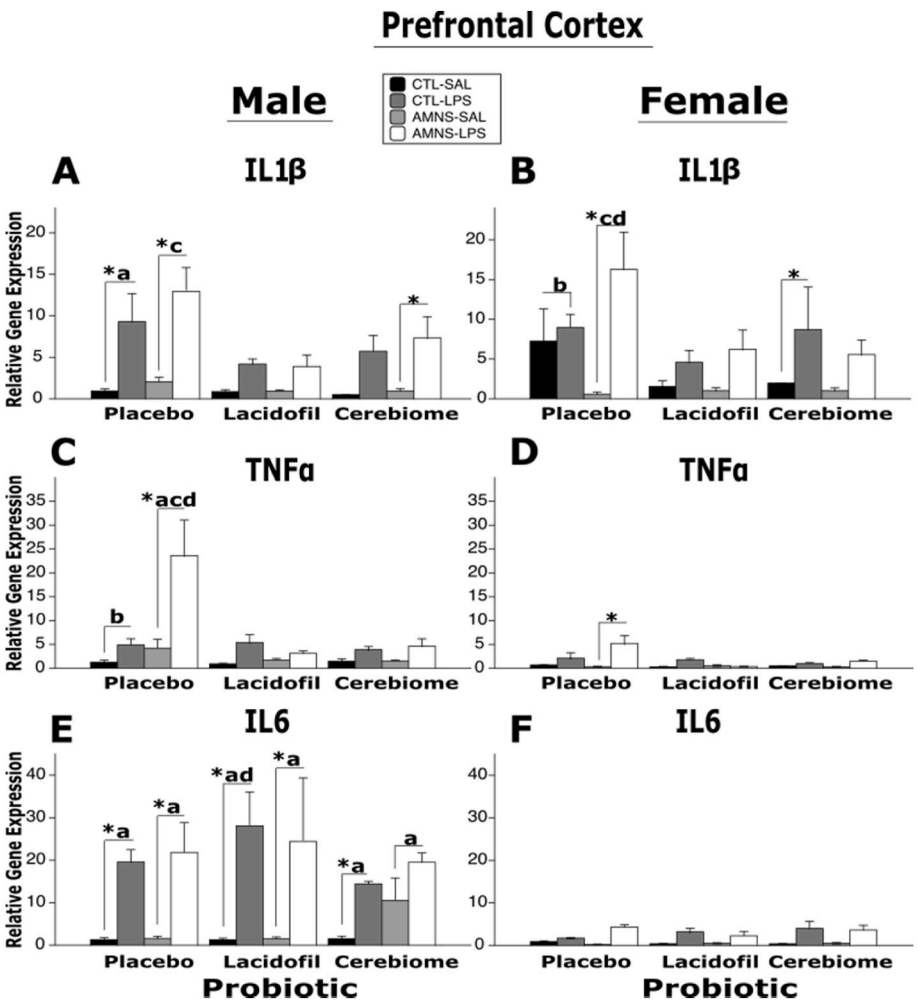

Fig. 5. Acute PFC (A) IL1β mRNA expression of males, (B) IL1β mRNA expression of females, (C) TNFα mRNA expression of males, (D) TNFα mRNA expression of female mice, (E) IL6 mRNA expression of males, and (F) IL6 mRNA expression of female six-week-old mice treated with saline (SAL) or LPS, water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), and supplemented with placebo, *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®), or *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®). Data represented as mean fold change (±SEM), n = 18–20/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (a) denotes a significant difference between male and female counterparts ($p < 0.05$), (b) denotes a significant difference between water and antimicrobial treatments (c) denotes a significant difference from the *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®) experimental condition ($p < 0.05$), and (d) denotes a significant difference from the *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®) experimental condition ($p < 0.05$).

0.01, $\eta_p^2 = 0.63$), IL12 ($F_{(1,89)} = 90.57$, $p < 0.01$, $\eta_p^2 = 0.50$), and TNFα ($F_{(1,108)} = 162.40$, $p < 0.01$, $\eta_p^2 = 0.65$). Significant antimicrobial x probiotic ($F_{(2,93)} = 6.07$, $p < 0.01$, $\eta_p^2 = 0.12$) and LPS x antimicrobial x probiotic ($F_{(2,93)} = 6.014$, $p < 0.01$, $\eta_p^2 = 0.12$) interactions were found for IFNγ. There were also trends towards a main effect of probiotic ($F_{(1,89)} = 2.93$, $p = 0.06$, $\eta_p^2 = 0.06$) and a LPS x probiotic interaction ($F_{(1,89)} = 2.94$, $p = 0.06$, $\eta_p^2 = 0.06$) for IL1β along with a trend towards a LPS x antimicrobial x probiotic interaction for IL12 ($F_{(2,89)} = 2.97$, $p = 0.06$, $\eta_p^2 = 0.06$).

Pairwise comparisons showed that regardless of sex, antimicrobial and probiotic treatments, LPS-treated mice displayed significantly greater IFNγ (*MD* = 1522.31, *SE* = 111.01, $p < 0.01$), IL1β (*MD* = 16.31, *SE* = 1.72, $p < 0.01$), IL6 (*MD* = 1404.15, *SE* = 151.11, $p < 0.01$), IL10 (*MD* = 158.78, *SE* = 12.84, $p < 0.01$; see Fig. 6G and H), IL12 (*MD* = 12.89, *SE* = 1.36, $p < 0.01$) and TNFα (*MD* = 29.83, *SE* = 2.34, $p < 0.01$; see Fig. 6K and L) concentrations compared to their saline-treated counterparts. LPS-treated males had significantly greater IL6 concentration compared to their female counterparts (*MD* = 711.49, *SE* = 210.78, $p < 0.01$; see Fig. 6E and F). Antimicrobial- and LPS-treated mice who received *L. rhamnosis* R0011 and *L. helveticus* R0052 treatment had significantly lower IL12 expression compared to their placebo-treated counterparts (*MD* = 11.47, *SE* = 3.08, $p < 0.01$; see Fig. 6I and J). Additionally, LPS-treated mice supplemented with placebo had significantly greater IL1β concentration compared to *L. rhamnosis* R0011 and *L. helveticus* R0052 treated (*MD* = 9.20, *SE* = 2.91, $p < 0.01$), and *L. helveticus* R0052 and *B. longum* R0175 treated (*MD* = 8.07, *SE* = 2.86, $p < 0.05$) counterparts (see Fig. 6C and D). Regardless of sex and LPS, pairwise comparisons showed that antimicrobial-treated mice supplemented with *L. rhamnosis* R0011 and *L. helveticus* R0052 had a significantly lower IFNγ concentration than *L. helveticus* R0052 and *B. longum* R0175 treated counterparts (*MD* = −616.03, *SE* = 190.55, $p < 0.01$). Further analysis showed that the antimicrobial-treated mice supplemented with *L. rhamnosis* R0011 and *L. helveticus* R0052 had significantly less IFNγ expression compared to their water-treated counterparts (*MD* = −540.63, *SE* = 198.78, $p < 0.01$); while antimicrobial-treated mice supplemented with *L. helveticus* R0052 and *B. longum* R0175 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 x antimicrobial x probiotic interaction for IFNγ and IL12 showed that water and LPS-treated mice that were supplemented with *L. helveticus* R0052 and *B. longum* R0175 had significantly lower IFNγ concentration compared to their placebo-treated counterparts (*MD* = −754.66, *SE* = 261.44, $p < 0.05$); while antimicrobial and LPS treated mice that were supplemented with *L. rhamnosis* R0011 and *L. helveticus* R0052 had significantly lower IL12 expression compared to placebo-treated counterparts (*MD* = 11.47, *SE* = 3.08, $p < 0.01$; see Fig. 6I and J). As well, there was a trend towards lower IFNγ concentration in the *L. helveticus* R0052 and *B. longum* R0175 group compared to their *L. rhamnosis* R0011 and *L. helveticus* R0052 treated counterparts (*MD* = −654.79, *SE* = 277.29, $p = 0.06$). In the antimicrobial-treated groups, LPS-treated mice had significantly less IFNγ concentration when supplemented with *L. rhamnosis* R0011 and *L. helveticus* R0052 than counterparts supplemented with *L. helveticus* R0052 and *B. longum* R0175 (*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

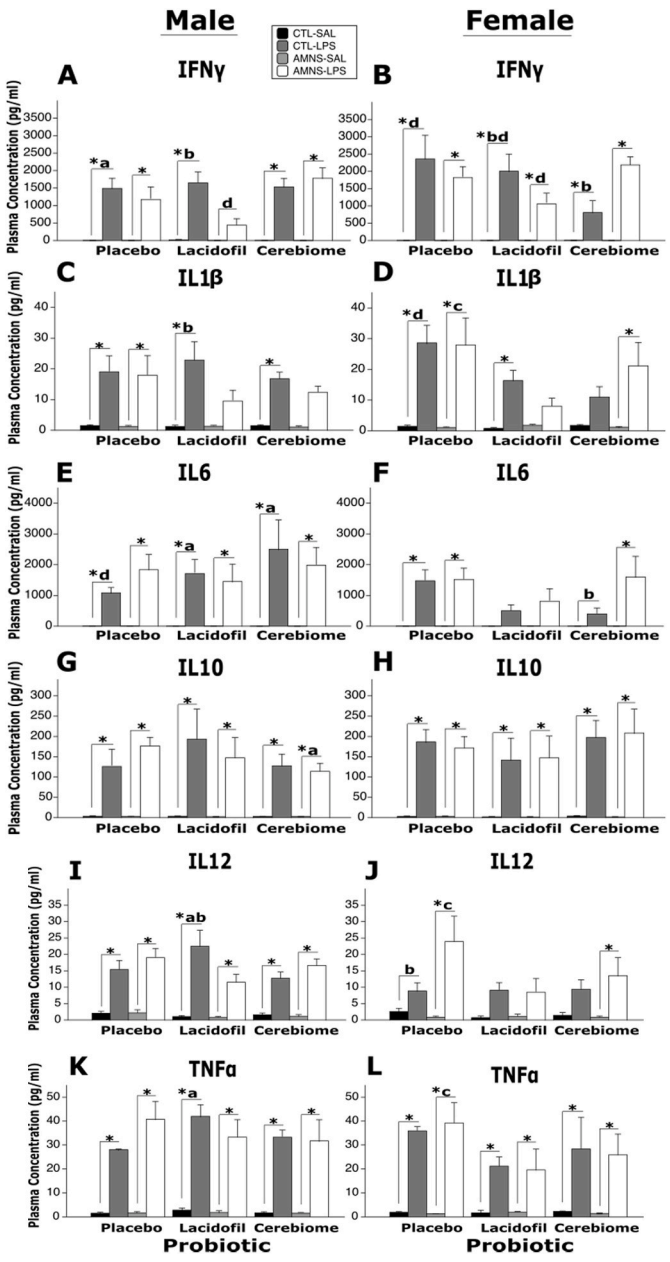

Fig. 6. Acute plasma IFNγ concentration of (A) males, and (B) females, acute plasma IL1β concentration of (C) males, and (D) females, acute IL6 plasma concentration of (E) males, and (F) females, acute plasma IL10 concentration of (G) males, and (H) females, acute plasma IL12 concentration of (I) males, and (J) females, and acute plasma TNFα concentration of (K) males, and (L) female six-week-old mice treated with saline (SAL) or lipopolysaccharide (LPS), water (CTL-SAL, CTL-LPS) or antimicrobials (AMNS-SAL, AMNS-LPS), and supplemented with either placebo, *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®), or *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®). Data represented as mean fold change (±SEM), n = 18–20/group. The asterisks (*) denotes a significant difference between LPS and saline counterparts ($p < 0.05$), (a) denotes a significant difference between male and female counterparts ($p < 0.05$), (b) denotes a significant difference between water and antimicrobial treatments, (c) denotes a significant difference from the *L. rhamnosis* R0011 and *L. helveticus* R0052 (Lacidofil®) experimental condition ($p < 0.05$), and (d) denotes a significant difference from the *L. helveticus* R0052 and *B. longum* R0175 (Cerebiome®) experimental condition ($p < 0.0$.

L. rhamnosis R0011 and *L. helveticus* R0052 had significantly greater IFNγ concentration than antimicrobial-treated counterparts ($MD = 1074.03$, $SE = 277.29$, $p < 0.01$), while LPS-treated mice exposed to water and *L. helveticus* R0052 and *B. longum* R0175 had significantly less IFNγ concentration compared to antimicrobial-treated counterparts ($MD = -814.00$, $SE = 269.48$, $p < 0.01$; see Fig. 6A and B).

4. Discussion

Immune challenges experienced during puberty can influence the maturation and reactivity of the immune system [30]. For example, exposure to an immune challenge or antimicrobials, in adults, can lead to short-term and long-term changes in immune reactivity [26,29]. However, research on the effect of antimicrobial and LPS treatments on acute immune responsivity in pubertal male and female mice was lacking. Thus, to our knowledge, this experiment is the first to investigate the impact of pubertal antimicrobial and LPS treatment on acute immune responsivity in male and female mice, and to determine if this effect can be mitigated by probiotics. Our results showed that pubertal antimicrobial and LPS treatment increased intestinal weights, sickness behaviours, neuroinflammation, and plasma cytokine concentrations while pubertal probiotic treatment successfully mitigated these effects.

Antimicrobial-treated mice had significantly higher whole intestinal weights compared to water-treated mice. Our results also showed that antimicrobial-treated mice exposed to LPS had significantly higher intestinal weights compared to their LPS- and water-treated counterparts. Intestinal weight changes have been used to confirm the broad effects of antimicrobials on the intestinal environment [49]. Increased intestinal weight is associated with significant colonic remodeling, reduced bacterial content, impaired digestive function, water retention, and increased intestinal inflammation [35,49]. Moreover, previous research has shown that ampicillin treatments in C57BL/6 mice reduces tight-junction proteins, and increases cecal weight, intestinal permeability and endotoxin concentrations in the intestinal lumen [39]. Therefore, increased intestinal weight in our mice may indicate colonic remodeling which could cause increases in intestinal permeability and intestinal inflammation along with a reduction in microbial diversity.

Antimicrobial treatment altered LPS-induced cytokine concentrations in the blood. Our results showed that mice exposed to antimicrobials had significantly greater LPS-induced IL12 concentrations in the plasma compared to water-treated mice. This result is consistent with our first hypothesis, as well as with previous research. Studies have consistently shown that antimicrobial treatments increase peripheral inflammation [27]. IL12 is a pro-inflammatory cytokine, that activates and regulates immune cells [43]. The increase of LPS-induced IL12 concentrations in antimicrobial-treated mice is likely due to disruptions of the intestinal barrier. Previous research has shown that antimicrobial treatments increase the translocation of microbes and bacterial endotoxins across the epithelial barrier into peripheral circulation [36]. Therefore, increased bacterial concentrations prior to LPS exposure may activate of TLR-expressing immune cells, and increase IL12 concentrations during an acute immune challenge [3].

Antimicrobial treatments also potentiated LPS-induced central inflammation. Our results showed that regardless of LPS treatment, antimicrobial-treated males displayed significantly greater TNFα mRNA expression in the PFC compared to their water-treated counterparts. This result was consistent with our third hypothesis, as well as with previous research [26]. TNFα is involved in pro-inflammatory processes and in cellular degeneration [44]. The increased expression of this neuro-degenerative marker in the PFC may have stemmed from antimicrobial-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 [11]. Chronic vagal activation can lead to increased firing rate of cholinergic neurons projecting from the basal ganglia, which lead to thalamic and primary sensorimotor regions [48].

Continuous neural excitation of these regions is associated with stress-induced alterations of NMDA-mediated calcium influx [46], pro-inflammatory cytokine production [12], oxidative markers [28] and apoptosis [41]. Therefore, increased degenerative markers in antimicrobial-treated mice may be due to chronic hyperactivity of cells in the PFC.

The influence of circulating hormones on immune cells could explain the sexually dimorphic effect of antimicrobials and LPS on TNFα and IL6 mRNA expression in the PFC of male mice. Previous research has shown that females have protective mechanisms against acute immune challenge [34]. Estradiol and progesterone are immune enhancers and have anti-inflammatory properties within the CNS, and have been shown to reduce neuro-degeneration, oxidative damage, and neuroinflammation in female mice [14], through the direct binding of estradiol to ER-α receptors on immune cells [34]. In contrast, testosterone has been shown to suppress immune function and promote neuroinflammation and pro-degenerative mechanisms [1]. Additionally, bioactive metabolites (i.e., equol, enterolignans, and urolithins) synthesized by gut bacteria from dietary compounds (i.e., phytoestrogens) have been shown to modulate hormone levels and have estrogenic effects [25]. Therefore, it is possible that females may be less susceptible to LPS and to antimicrobial-induced neuroinflammation, due to the mitigating effects of estradiol on the immune system. Our results also show that female mice treated with LPS had significantly greater hippocampal IL1β and IL6 mRNA expression in comparison to their LPS-treated male counterparts. Increased IL1β and IL6 mRNA expression in the hippocampus of females may be indicative of an increased susceptibility to developing depression in adulthood. Previous research has shown an association between IL1β and IL6 cytokine expression and depression [21]. Moreover, LPS-treated female mice have been shown to be more susceptible to developing depression in adulthood compared to their male counterparts [29]. Therefore, these acute increases of hippocampal IL1β and IL6 mRNA expression in female mice may be a contributing factor in the development of depression in adulthood.

Probiotic supplementation was associated with reduced peripheral inflammation, but in a cytokine-specific manner. In water-treated mice, *L. helveticus* R0052 and *B. longum* R0175 supplementation reduced LPS-induced IL1β and IFNγ concentrations in the plasma, compared to placebo-treated counterparts. *L. rhamnosis* R0011 and *L. helveticus* R0052 supplementation reduced LPS-induced IL1β plasma concentrations compared to placebo-treated counterparts. *L. rhamnosis* R0011 and *L. helveticus* R0052 supplementation in antimicrobial-treated mice was also found to significantly reduce LPS-induced IL12 and IFNγ plasma concentrations compared to placebo-treated mice. These results are consistent with our second hypothesis, and with previous research [9, 30]. IL1β is a pro-inflammatory cytokine produced through NLRP3-inflammasome activation [8], and is involved in up-regulating NF-κβ-induced pro-inflammatory cytokines [32]. Similarly, IFNγ potentiates pro-inflammatory signaling through alterations of NLRP3-inflammasome activity [24]. It is theorized that probiotic supplementation reduces pro-inflammatory cytokine production through the secretion of bioactive molecules that enter systemic circulation and influence metabolic pathways [47]. Research in HT-29 intestinal epithelial cells treated with *Salmonella enterica* serovar Typhimurium secretome, TNFα and the *L. rhamnosus* R0011 secretome (LrS) has shown that LrS induces the expression of dual specificity phosphatase 1, activating transcription factor 3, and tribbles pseudokinase 3, negative regulators of the NF-κB and MAPK pathways [18]. Other research using male adult Flinder Sensitive Line rats (rodent model of depression) demonstrated that treatment with *L. helveticus* R0052 and *B. longum* R0175 influenced one-carbon and catecholamines metabolism by increasing S-adenosylmethionine in the liver and decreasing plasma betaine, dopamine, and norepinephrine expression [42]. Therefore, differences in bioactive molecules secreted by L. *rhamnosus* and *L. helveticus* may explain the observed differences in cytokine production between *L. rhamnosis* R0011 and *L. helveticus* R0052 and *L. helveticus* R0052 and *B. longum* R0175.

Alterations of antimicrobial-induced peripheral inflammation by *L. rhamnosis* R0011 and *L. helveticus* R0052 was also associated with reductions in sickness behaviours. The results showed that regardless of LPS or saline treatment, antimicrobial-treated mice supplemented with *L. rhamnosis* R0011 and *L. helveticus* R0052 displayed significantly fewer sickness behaviours compared to placebo and *L. helveticus* R0052 and *B. longum* R0175 treated counterparts. The result was partially consistent with our second hypothesis and with previous research. Research with male BALB/c mice exposed to a *Helicobacter pylori* infection demonstrated that *L. rhamnosus* R0011 and *L. helveticus* R0052 treatment significantly increases the rate of recovery of host pathophysiology through the reduction of chronic gastric inflammation along with improving gastric motor function and intestinal permeability [45]. It is theorized that the reduction of peripheral cytokine concentrations in antimicrobial-treated mice exposed to *L. rhamnosis* R0011 and *L. helveticus* R0052 reduced the convergence of inflammatory signals to the lower brain regions, thus reducing cytokine-induced neural activation of thalamic regions implicated in sickness behaviours.

Reduced peripheral LPS-induced cytokine expression was also associated with alterations in cytokine mRNA expression in the hippocampus and PFC. Male and female mice supplemented with *L. rhamnosis* R0011 and *L. helveticus* R0052 or *L. helveticus* R0052 and *B. longum* R0175 prior to LPS exposure had significantly lower LPS-induced hippocampal IL6 and PFC IL1β mRNA expression, compared to their placebo-treated counterparts. Moreover, male mice treated with *L. rhamnosis* R0011 and *L. helveticus* R0052 or *L. helveticus* R0052 and *B. longum* R0175 displayed reduced PFC TNFα mRNA expression in comparison to their placebo-treated counterparts. These results supported our second and fourth hypotheses, and are consistent with previous research. Studies from our laboratory have shown that two weeks of probiotic supplementation significantly reduced hippocampal IL6, IL1β, and TNFα mRNA expressions after LPS exposure [30]. IL6 is a cytokine involved in both pro-inflammatory (JAK/STAT) and anti-inflammatory (MAPK) mechanisms, as well as the regulation of cellular metabolism, regeneration, and neural processes [37]. It is theorized that reductions in plasma cytokine concentrations in probiotic 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 the thalamic regions, thus reducing microglial mediated up-regulation of IL6, TNFα, and IL1β mRNA expression, compared to the placebo-treated counterparts.

Notably, the effect of the probiotics on LPS-induced TNFα mRNA expression in the PFC was limited to males. Males exposed to LPS displayed significantly higher TNFα mRNA expression in the PFC compared to females, and supplementation with *L. rhamnosis* R0011 and *L. helveticus* R0052 or *L. helveticus* R0052 and *B. longum* R0175 significantly reduced TNFα mRNA expression. The sexually dimorphic effects of probiotics on central inflammation may be due to sex differences in the gut microbiota earlier in life. The onset of puberty in NOD/ShiLtJ males is associated with a reduction in microbial diversity, while pubertal females maintain higher microbial diversity into adulthood [33]. Research in BALB/c and C57BL/6 mice has shown that bacterial strains such as *Lactobacillus plantarum*, *Bacteroides distasonis*, and *Bifidobacterium* were higher in females compared to male mice [9]. Therefore, probiotic supplementation may confer more benefits in males, due to their reduced microbial diversity and lower proportions of *Lactobacillus* and *Bifidobacterium* strains.

4.1. Limitations and future directions

There are ongoing debates about whether the gavage treatment is stressful to mice. Studies have shown that a single gavage in rats elevated plasma corticosterone levels 4 h post-treatment [4]. However, other researchers have found no significant difference in plasma

corticosterone concentration in mice [19]. Further research should consider a control non-gavage group to determine if there is an effect of the gavage procedure on stress and immune reactivity. Secondly, considering the acute nature of this study, we cannot extrapolate the findings to brain function and behavior outside of this timeframe. Further research is required to assess the long-term effects of LPS and antimicrobial treatments on brain function and behavior. Lastly, the stressor used in this experiment (i.e. LPS) specifically stimulates toll- like receptor 4, therefore, broad conclusions regarding stress cannot be made with this current research design. Further research should consider using an alternative stressor to determine the effects of other stress-related pathways on immune responsivity during puberty. Further research should also examine the effects of LPS and antimicrobial treatments on microbial composition to confirm whether gut dysbiosis is a contributing factor to the observed effects in this study.

5. Conclusions

Overall, this study shows that pubertal antimicrobial and LPS treatments significantly affect acute immune-responsivity and that probiotic supplementation mitigates these effects. Pubertal antimicrobial treatment aggravates LPS-induced pro-inflammatory immune response, while probiotic supplementation mitigates this effect in a sex-specific manner. *L. rhamnosis* R0011 and *L. helveticus* R0052 supplementation is more effective at reducing the pro-inflammatory response in pubertal mice treated with antimicrobials, while *L. helveticus* R0052 and *B. longum* R0175 supplementation is more effective in pubertal mice who were not treated with antimicrobials. The current study is the first to assess the effects of pubertal probiotic and antimicrobial treatments on LPS-induced acute immune response.

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Declaration of interests

None.

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