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# Comprehensive Psychoneuroendocrinology

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## Adolescent use of potential novel probiotic *Rouxiella badensis* subsp. *acadiensis* (*Canan SV-53*) mitigates pubertal LPS-Induced behavioral changes in adulthood in a sex-specific manner by modulating 5HT1A receptors expression in specific brain areas

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### ARTICLE INFO

#### Keywords:

Probiotic  
Depression  
Anxiety  
5HT1A  
Hippocampus  
Raphé-nuclei

### ABSTRACT

Adolescence is a critical period of development during which the brain undergoes significant remodeling that impacts behavior later in life. Exposure to stress, and especially immune challenge, during this period triggers changes in brain function resulting in the development of mental disorders in adulthood, such as depression and anxiety. Previous studies from our laboratory have shown that a single exposure to LPS (lipopolysaccharide) during puberty causes enduring depression-like behaviour in females and anxiety-like behaviours in males. However, administration of probiotics during puberty blocked the enduring effects of LPS on depression-like and anxiety-like behaviors in female and male mice, respectively. These results suggest that the gut microbiome is a mediator of the effects of stress on mental health. The objective of the current study is to examine the effectiveness of a novel probiotic *Rouxiella badensis* subsp. *acadiensis* (*Canan SV-53*) in blocking LPS-induced anxiety-like and depression-like behaviors in adult male and female mice. Our results showed that *Rouxiella badensis* subsp. *acadiensis* (*Canan SV-53*) blocked LPS-induced depression-like behavior in female mice. We also found that pubertal treatment with *Rouxiella badensis* subsp. *acadiensis* (*Canan SV-53*) mitigated the LPS-induced decrease in 5HT1A expression in CA1 as well as the LPS-induced increase in 5HT1A expression in the raphe-nuclei in female mice. Contrary to our predictions, pubertal LPS treatment at 6 weeks of age did not induce enduring anxiety-like behavior in males. There was also no difference in anxiety-like behavior between the LPS-sucrose and LPS-probiotic male groups. However, pubertal LPS treatment increased the expression of 5HT1A receptors in the DRN in males, while probiotic exposure mitigated this increase. Our study highlights the consequences of stress exposure (immune challenge) on mental health in adulthood taking into consideration 5HT1A receptors expression at different regions of the brain. It also emphasizes on the importance of considering adolescence as window of opportunities during which probiotic use can alleviate the long-term neural and behavioral alterations induced by stress.

### 1. Introduction

Adolescence is a critical period of development that stretches between childhood and adulthood and it is defined as social, cognitive,

emotional and reproductive maturation [1]. This period is marked by brain remodeling and reorganizing which subsequently impact social cognitive behavior [2]. A consequence of this major neuronal transformation and structural reshaping of neural circuits is an increased

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<https://doi.org/10.1016/j.cpnec.2021.100063>

Received 18 March 2021; Received in revised form 31 May 2021; Accepted 1 June 2021

Available online 5 June 2021

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degree of vulnerability to stressors and immune challenges [3]. More specifically, exposure to stress during adolescence results in short-term and long-term changes in brain neurochemistry, physiology and behavior in adulthood [4,5]. Thus, exposure to certain stressors during this critical period of development can increase the likelihood of developing mental illnesses like depression and anxiety later in life.

The immune system is also involved in the pathophysiology of anxiety and depression. In the past decade, numerous studies have shown higher risk for low-grade inflammation and greater concentrations of pro-inflammatory markers like interleukin 6 (IL-6), tumor necrosis factor alpha (TNF $\alpha$ ) and c-reactive protein in the blood of depressed and anxious patients [6]. The severity of anxiety symptoms is positively associated with blood concentration of several pro-inflammatory cytokines [6]. Moreover, recent research from our laboratory shows that a single injection of lipopolysaccharide (LPS), a gram negative bacterial endotoxin, at 6 weeks old, a pubertal stress-sensitive period, induces enduring depression-like behaviour in female mice and anxiety-like behaviour in male mice [7]. Anxiety and depression are associated with a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and disturbances in the autonomic nervous system [8,9]. The activation of the HPA axis and the release of glucocorticoids play a key role in modulating inflammation and cytokine response. This cross-talk between the nervous, endocrine, and immune systems serves to maintain physiological homeostasis during inflammatory and stress responses that induce systemic cytokine production [9]. Furthermore, several studies have demonstrated the ability of acute lipopolysaccharide LPS injection to stimulate the hypothalamic-pituitary-adrenal HPA axis and to increase corticosterone concentration in the blood [10–12].

Serotonin or 5-hydroxytryptamine (5HT) is one of several monoamine neurotransmitters that contributes to the pathogenesis of anxiety and depression with at least 14 serotonin receptor subtypes involved. 5-HT $1A$  receptors are of primary importance as they are targets for a number of antidepressants and anxiolytic treatments [13]. These receptors exist both at presynaptic (autoreceptors) and postsynaptic sites (heteroreceptors), with 5-HT $1A$  autoreceptors being located deep within the brainstem and 5-HT $1A$  heteroreceptors localized in various brain regions including the hippocampus, prefrontal cortex, thalamus, lateral septum, amygdala, and hypothalamic nuclei [14]. Post mortem analyses in depressed patients revealed an increase in 5HT $1A$  autoreceptor expression in the dorsal raphe nuclei (DRN) [15] and a decrease in 5HT $1A$  heteroreceptor expression in the hippocampus [16,17]. Exposure to chronic stress has also been found to alter 5HT $1A$  receptor expression in the hippocampus in rodent models of depression and anxiety [18,19]. For example, exposure to prolonged maternal separation causes a decrease in hippocampal 5HT $1A$  mRNA expression and an increase in depression-like behavior in rats [20]. Likewise, 5HT $1A$  knockout mice display greater anxiety-like behavior than do wild-type counterparts [21].

Numerous studies have also linked intestinal dysbiosis to anxiety and depression [22]. There is a bidirectional relationship between the gut and the brain called the gut-brain axis. The gut-brain axis plays a vital role in maintaining homeostasis and involves complex crosstalk between the endocrine, immune and the autonomic nervous systems [22]. In healthy adults, 90% of the gut microbiota is dominated by the Bacteroidetes and Firmicutes phyla. In contrast, in depressed individuals, there is an alteration in the abundance of different genera within the Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria phyla [23]. Exposure to certain stressors like maternal separation, crowding, heat and acoustic stress can harmfully affect the composition of the gut microbiota [24]. However, further research is needed to decipher causality and the importance of microbiota in increasing or decreasing inflammation in the gut and in the brain. Nevertheless, recent microbiome research suggests a key role of the gut microbiota in brain development, behaviour and mood [25]. The gut microbiome is also important for mental health and for shaping behavioral responses [26].

Probiotics are live microorganisms that confer a health benefit on the

host, when administered in adequate amounts [27]. They can mitigate the stress response and have anti-depressive and anxiolytic effects by modulating inflammatory responses [7,28,29]. The interaction of the microbiota with other environmental risk factors, such as stress and diet, opens new horizons in the development of interventions (like probiotics use) targeting the gut microbiota for the prevention and treatment of mental health disorders [25]. Recent findings from our laboratory demonstrate that consumption of the probiotic *Lactobacillus reuteri* throughout pubertal development mitigates LPS-induced enduring effects on memory dysfunction, anxiety-like behaviour and stress reactivity in adulthood. Moreover, pubertal exposure to probiotics from kefir blocks LPS-induced depression-like behaviour in females and anxiety-like behaviors in males. Although several studies support the benefit of probiotic interventions in mental health and behavioural modifications, the neurochemical changes or the mechanisms underlying these effects remain unclear. It is also unknown whether our previous findings would extend to other probiotics [29,30]. LPS is a pyrogenic compound present in the outer membrane of gram-negative bacteria. It activates the innate immune system by binding to toll-like receptors (TLRs) located on innate immune cells (e.g. macrophages, monocytes) [31]. In the brain, these receptors are present on meninges endothelial cells and microglia [31]. TLR-4 is the main receptor involved in the activation of a series of downstream pathways and transcription factors (such as NF- $\kappa$ B) that induce the production of proinflammatory (e.g. interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor (TNF)- $\alpha$ ) or anti-inflammatory (e.g. IL-10) immunomodulating cytokines [32,33]. Central or peripheral administration of LPS can induce neuroinflammation [34]. Peripheral administration of LPS can cause an inflammatory response in the CNS, either by stimulating *de novo* synthesis of cytokines within the brain [35–37] or by increasing the permeability of the blood-brain barrier (BBB) and permitting infiltration of inflammatory cells such as mast cells, cytokines and chemokines into the brain [37,38] which contribute to the development of sickness symptoms and behavioral changes [46,54].

Therefore, the current study investigated whether the use of a novel probiotic bacterium "*Rouxiella badensis* subsp. *acadiensis*" during puberty can block LPS-induced depression-like and anxiety-like behaviors in adult female and male mice. *Rouxiella badensis* subsp. *acadiensis* has been filed in a U.S. Provisional Application No. 62/916,921 entitled "Probiotics Composition and Methods" for its potential probiotic effects [39]. *Rouxiella badensis* subsp. *acadiensis* is a bacterium isolated from the microbiota of the wild blueberry fruit. Based on its whole-genome shotgun sequencing, there are no pathogenicity genes associated to it [39,66]. The bacterium has been used in multiple studies and it was found to be an effective anti-inflammatory agent for neurodegeneration, diabetes, and cancer [41–45]. Moreover, *Rouxiella badensis* subsp. *acadiensis* can modulate gut-associated non-specific immunity without an inflammatory outcome [40].

Our study assesses the long-term behavioral impacts of the selected bacterium as a probiotic and its relationship with the expression of 5HT $1A$  receptor in a sex-specific manner. We hypothesize that consumption of *Rouxiella badensis* subsp. *acadiensis* during pubertal development can block LPS-induced enduring depression-like behavior by modifying the expression of 5HT $1A$  receptors in the raphe nuclei and in the hippocampus in adulthood.

## 2. Methods

### 2.1. Animal housing

Ninety-six CD-1 mice (48 male and 48 female) were shipped from Charles River Laboratories (St-Constant, Québec) at 3 weeks of age. Mice were housed in pairs in Polycarbonate cages either in an all-male or an all-female colony room with *ad libitum* access to food (ENGIVO—Teklad Global 18% rodent) and water. Housing rooms were kept at constant temperature (24  $\pm$  2  $^{\circ}$ C) with a reversed light: dark cycle (14:10; lights

off at 10:00am) and 40% ( $\pm 5\%$ ) humidity. Dusk and dawn were gradually induced over a period of 1 h. Cages were bedded with Teklad Corn Cob (Harlan Laboratories, Inc., Madison, WI, US,  $\frac{1}{4}$  inches in diameter). Enrichment in the cages consisted of one square piece of Nestlet (Ancare Corp., Bellmore, NY, US) and a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, CA). All procedures were approved by the Animal Care Committee of the University of Ottawa.

## 2.2. Probiotics

Stock cultures of the *Rouxiiella badensis* subsp. *acadiensis* were maintained at  $-80\text{ }^{\circ}\text{C}$  in Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, USA) supplemented with 30% (v/v) glycerol. The bacterium was grown in TSB at  $30\text{ }^{\circ}\text{C}$  for 20 h. From 5 to 7 weeks of age, mice in the experimental group received  $10^9$  CFU of the probiotic in 1% sucrose dissolved in 1X PBS pH7.4 through oral gavage. Control mice received the same volume of 1% sucrose in 1X PBS by oral gavage. Fresh probiotic and 1% sucrose solutions were prepared daily.

## 2.3. Lipopolysaccharide (LPS)

Lyophilized LPS (obtained from *Escherichia coli* serotype O26:B6; #L3755; Sigma Aldrich) was dissolved into 0.9% (w/v) sterile saline (Ricca Chemical Company) at a concentration of 0.2 mg/ml. At 6 weeks of age, all mice were injected intraperitoneally at the end of the light phase with either 0.9% sterile saline or LPS (1.5 mg/kg). This dose of LPS has previously been shown to cause mild sickness in mice for up to 48 h and to produce enduring impairments in reproductive and non-reproductive behaviors, when administered at 6 weeks of age, during the stress-sensitive pubertal period [5,67,68].

## 2.4. Sickness behaviors

Sickness behaviors were monitored at 30 min, 2, 4, 8, 12, 24 and 48 h following injection by two blind raters. The assessment was carried out using the protocol described in Kolmogorova et al. (2017) [46]; a four-point scale was used to assess mice for the presence of four sickness behaviors (lethargy, huddling, ptosis, and piloerection) at specific time points following exposure to LPS. Scores ranging from 0 to 4 were assigned based on the number of symptoms displayed at each time point. Scores were averaged between the two raters.

## 2.5. Body weight

Mice were weighed at 5 weeks of age (before the start of the probiotic or control treatment), at 6 weeks of age (before the saline or LPS injection), at 24 h and 48 h after saline or LPS injection, at 7 weeks of age (upon termination of the probiotic or control treatment), and at 10 weeks of age (in adulthood).

## 2.6. Behavioural testing

At 10 weeks of age, mice were exposed to a battery of behaviour tests to examine anxiety-like and depression-like behaviors. Each test ran over two consecutive days and all mice were exposed to the behavior tests in the same order. Testing began 2 h after the start of the dark phase and was conducted under dim red light. All testing was completed within 3.5 h each day. To evaluate anxiety-like behavior, the Elevated Plus Maze (EPM) and Open Field Test (OFT) were used, whereas depression-like behavior was assessed using the Forced Swim Test (FST) and the Tail Suspension Test (TST). All mice were given a 48-h rest period between the behavior tests to avoid any carry-over effects. Panasonic WV-CP284 camera mounted on an Optex T165 tripod and an Ethovision Tracking software were used for tracking and recording.

### 2.6.1. Anxiety-like behaviour testing

**2.6.1.1. Open field test (OFT).** The open field consisted of a square-shaped wall-enclosed area. The open field (100 cm  $\times$  100 cm) was divided into a central/inner zone (30 cm  $\times$  30 cm) and a peripheral/outer zone (100 cm–30cm). During the dark phase of their light cycle, mice were placed in the open field for 5 min. The total distance travelled, time spent in inner zone, latency to first enter inner zone and time spent in outer zone were measured using the Ethovision Tracking software. Less time spent in the inner zone of the maze is associated with greater anxiety [47].

**2.6.1.2. Elevated plus maze (EPM).** The Elevated Plus Maze (EPM) consisted of four branching arms: two open arms and two closed arms. Each arm is 10 cm  $\times$  50 cm. During the test, mice were placed in the center facing one of the open arms for 5 min to explore the maze. The Ethovision Tracking software was used to measure the amount of time spent in each portion of the maze throughout the 5-min trial. An increased amount of time spent in the closed arms of the maze is linked to greater anxiety [48].

### 2.6.2. Depression-like behavior testing

**2.6.2.1. Forced swim test (FST).** Mice were placed in a 4 L glass cylinder (15 cm (diameter)  $\times$  30 cm (height)) filled with 3 L of water at a temperature of  $24\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$  and allowed to swim for 5 min. The total time spent climbing (vertical movements along the wall of the cylinder), swimming (active circular swimming) and immobile (absence of movement or floating) was measured by two raters blind to treatment conditions. Inter-rater reliability factor was within 25%. The results were calculated as the average of the two raters' scores. At the end of the test, mice were removed from the glass cylinder and placed in a recovery cage on a heating pad until they were dry, and then they were returned to their home cages. Increased duration of immobility on the FST is an indication of increased behavioural despair and depression [49,50].

**2.6.2.2. Tail suspension test (TST).** Mice were suspended 60 cm above the ground within 1 cm from the tip of the tail for 5 min. The duration of immobility (absence of any body movement: the mice hung passively and motionless for at least 2 s) was measured by two raters blind to treatment conditions. Inter-rater reliability factor was within 25%. The results were calculated as the average of the two raters' scores. Mice were returned to their home cage upon termination of the testing. Increased duration of immobility on the TST is an indication of increased behavioural despair and depression [51].

## 2.7. Tissue collection and immunohistochemistry (IHC)

Mice were intracardially perfused with 20 ml of 0.9% saline followed by 20 ml of 4% paraformaldehyde (PFA). Brains were extracted and post-fixed in 4% PFA for 2 h then placed in fresh 30% sucrose solution 2 and 24 h after post-fixing and stored at  $4\text{ }^{\circ}\text{C}$  until slicing. Brains were sliced at a thickness of 40  $\mu\text{m}$  into 4 series using an automated Leica VT1200 vibratome. Sections were kept in cryoprotectant at  $-20\text{ }^{\circ}\text{C}$ . Free-floating brain sections from one of the series from each mouse were rinsed in 1X TBS, incubated in antigen retrieval solution (0.05 M sodium citrate in 1X TBS) for 30 min, rinsed again with TBS and incubated in 0.1 M Glycine/1X TBS for 30 min. Following additional TBS rinses, sections were transferred for 30 min in a concentrated blocking solution containing TBS, 20% normal goat serum, 0.3% Triton-X, and 1% H<sub>2</sub>O<sub>2</sub> then incubated for 20 h at  $4\text{ }^{\circ}\text{C}$  into a solution containing a primary rabbit antibody specific to 5HT1A (ab227165 Abcam; 1:300, Cambridge, MA). Sections were then incubated for 60 min at room temperature in a secondary biotinylated goat anti-rabbit antibody (Vector, BA-1000, IgG; PC38 Millipore 1:500), rinsed for 30 min with 0.2% Triton-X

in TBS followed by 60 min incubation in the ABC detection system (Vector, Vectastain ABC kit Elite Pk-6100 standard; Vector Laboratories, Burlingame, CA, USA). Finally, freshly prepared diaminobenzidine (DAB) solution (DAB Substrate kit, SK-4100 Vector Laboratories) was used to stain targeted 5-HT1A receptors.

## 2.8. Image analysis and cell quantification

The Mouse Brain Atlas was used to locate the various brain regions [52]. The cornu-ammonis (CA) CA1 and CA3 of the hippocampus (i.e., bregma: -2.18 mm) and DRN (i.e., bregma -4.48 mm) were examined for 5HT1A receptor (5HT1A Rc) expression based on one representative section for each mouse. Images were captured at 10X using an Olympus BX51 light microscope connected to a Jenoptik ProRes MF scan camera. Kolor Autopano was used for stitching. Two raters blind to treatment conditions quantified the number of cells positive for receptors in specific brain regions using unbiased counting on the ImageJ software (version 1.48) with a coefficient of variation  $\leq 15\%$ .

## 3. Procedures

Ninety-six mice arrived at 3 weeks of age. At 5 weeks of age, mice were either exposed to sucrose vehicle (1% sucrose by gavage) or to *Rouxiella badensis* subsp. *acadiensis*. (in 1% sucrose by gavage) for two weeks. At 6 weeks of age, mice were injected with either saline or LPS. Mice were then monitored for sickness behaviour over the next 48 h. At 10 weeks of age, mice began behavior testing with EPM, OFT, FST and TST. A 48-h rest period separated each behavior test to reduce carry-over effects. Mice were euthanized following the completion of all behavior tests.

## 4. Statistical analyses

IBM SPSS V26 software was used to perform statistical analyses. All data were initially screened for outliers using boxplots generated by SPSS. In order to reduce the effects of extreme outliers, cases that fell outside the 1.5 interquartile range were adjusted using winsorization [53]. A four-way mixed ANOVA was used to examine the within-subject effect of time (2, 4, 8, 12, 24 and 48 h), and the between-subject effects of sex, probiotic treatment (control or probiotic *Rouxiella badensis* subsp. *acadiensis*) and immune challenge (saline or LPS) on sickness responses [29,46,54]. Three-way ANOVAs were used to examine the effects of sex, immune challenge and probiotic treatment on the duration of immobility, swimming and climbing in the FST, the duration of immobility in TST, time spent in the inner zone, latency to first enter inner zone, velocity and total distance travelled in the OFT, time spent in the open arms in the EPM, and 5HT1A Rc (s) expression in the CA1 and CA3 regions of the hippocampus and in the DRN. Significant interactions were followed by pairwise comparisons and a Bonferroni correction factor was applied to control for multiple comparisons. The criterion for statistical significance was set at  $p < 0.05$ .

## 5. Results

### 5.1. Sickness behaviors and weight changes

#### 5.1.1. Pubertal probiotic treatment reduced LPS-induced sickness behaviour in males and females in a time-specific manner

Four-way mixed ANOVA revealed main effects of time ( $F_{(3.9, 220.7)} = 117.05, p < 0.001, \eta_p^2 = 0.68$ ), LPS treatment ( $F_{(1, 56)} = 593.11, p < 0.001, \eta_p^2 = 0.91$ ), and probiotic treatment ( $F_{(1, 56)} = 23.93, p < 0.001, \eta_p^2 = 0.30$ ). There were also significant time  $\times$  probiotic ( $F_{(3.9, 220.7)} = 3.46, p < 0.01, \eta_p^2 = 0.06$ ), time  $\times$  LPS ( $F_{(3.9, 220.7)} = 131.88, p < 0.001, \eta_p^2 = 0.70$ ), probiotic  $\times$  LPS ( $F_{(1, 56)} = 23.92, p < 0.001, \eta_p^2 = 0.30$ ), time

$\times$  sex  $\times$  probiotic ( $F_{(3.9, 220.7)} = 3.54, p < 0.01, \eta_p^2 = 0.06$ ), and time  $\times$  sex  $\times$  probiotic  $\times$  LPS ( $F_{(3.9, 220.7)} = 2.98, p < 0.05, \eta_p^2 = 0.06$ ) interactions (Fig. 1 (i)).

Pairwise comparison revealed that, as expected, male and female mice treated with LPS displayed more sickness behaviors than saline controls (mean difference (MD) = 1.90, standard error (SE) = 0.11,  $p < 0.001$ ; MD = 1.80, SE = 0.11,  $p < 0.001$ ; respectively). However, pairwise comparisons of sex  $\times$  probiotic  $\times$  LPS interaction showed that LPS-treated male and female mice exposed to probiotics displayed less sickness behaviour than LPS-treated mice exposed to sucrose (MD = -0.54, SE = 0.15,  $p < 0.05$ ; MD = -0.95, SE = 0.15,  $p < 0.001$ ; respectively). Moreover, LPS-treated males exposed to probiotic showed more sickness behaviors than LPS-treated female counterparts (MD = 0.33, SE = 0.15,  $p < 0.05$ ). LPS-treated female mice exposed to sucrose displayed significantly more sickness behavior than LPS-treated females exposed to *Rouxiella badensis* subsp. *acadiensis* probiotic at 30min (MD = 0.625, SE = 0.14,  $p < 0.001$ ), 2 h (MD = 1.375, SE = 0.34,  $p < 0.001$ ), 4 h (MD = 1.44, SE = 0.24,  $p < 0.001$ ), 6 h; (MD = 1, SE = 0.25,  $p < 0.001$ ), 8 h (MD = 1.125, SE = 0.24,  $p < 0.001$ ), 12 h (MD = 0.875, SE = 0.29,  $p < 0.001$ ), and 24 h (MD = 1.125, SE = 0.28,  $p < 0.001$ ) following LPS treatment. Similarly, LPS-treated male mice exposed to sucrose displayed more sickness than LPS-treated males exposed to *Rouxiella badensis* subsp. *acadiensis* probiotic at 30min (MD = 0.875, SE = 0.14,  $p = 0.001$ ), 6 h (MD = 0.875, SE = 0.25,  $p = 0.001$ ), 8 h (MD = 0.81, SE = 0.24,  $p = 0.001$ ), 12 h (MD = 1, SE = 0.29,  $p = 0.001$ ) and 24 h (MD = 0.94, SE = 0.28,  $p < 0.01$ ) following LPS treatment.

#### 5.1.2. Probiotic failed to mitigate LPS-induced weight loss

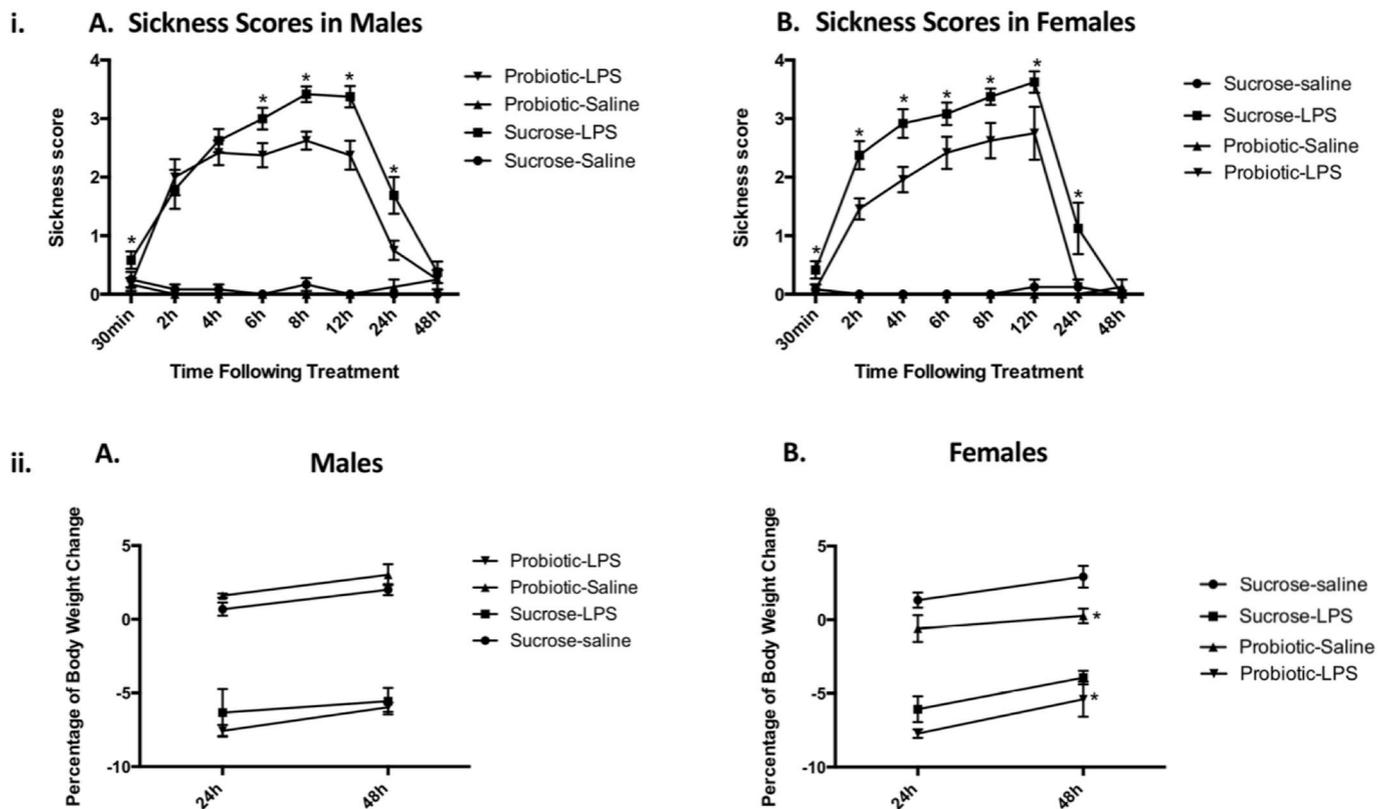
Within subjects tests showed a main effect of time ( $F_{(1.5, 82.7)} = 301.58, p < 0.001, \eta_p^2 = 0.85$ ) and a time  $\times$  sex interaction ( $F_{(1.5, 82.7)} = 8.33, p < 0.01, \eta_p^2 = 0.13$ ). Tests between subjects revealed a main effect of sex ( $F_{(1, 55)} = 6.6, p < 0.05, \eta_p^2 = 0.1$ ); LPS ( $F_{(1, 55)} = 20.49, p < 0.001, \eta_p^2 = 0.27$ ) and sex  $\times$  probiotic interaction ( $F_{(1, 55)} = 4.93, p < 0.05, \eta_p^2 = 0.08$ ). Pairwise comparison showed that, LPS-treated male mice exposed to sucrose displayed significant weight loss when compared to their saline counterparts at 24 h (MD = 7.016, SE = 1.09,  $p < 0.001$ ), 48 h (MD = 7.783, SE = 1.47,  $p < 0.001$ ), and 1week post-injection (MD = 3.406, SE = 1.36,  $p < 0.05$ ). Similarly, LPS induced weight loss in females control group at 24 h (MD = 7.401, SE = 1.09,  $p < 0.001$ ), 48 h (MD = 7.261, SE = 1.47,  $p < 0.001$ ), and 1week post-injection (MD = 4.401, SE = 1.36,  $p < 0.01$ ). Males treated with *Rouxiella badensis* subsp. *acadiensis* and injected with LPS demonstrated more weight loss than saline treated at 24 h (MD = 9.174, SE = 1.09,  $p < 0.001$ ), 48 h (MD = 10.164, SE = 1.47,  $p < 0.001$ ), and 1week post-injection (MD = 5.018, SE = 1.36,  $p < 0.05$ ) while females treated with *Rouxiella badensis* subsp. *acadiensis* and injected with LPS demonstrated more weight loss than saline treated at 24 h (MD = 7.124, SE = 1.13,  $p < 0.001$ ), 48 h (MD = 6.086, SE = 1.52,  $p < 0.001$ ) (Fig. 1 (ii)).

### 5.2. Probiotic treatment during puberty prevented LPS-induced depression-like behaviour in female mice during adulthood

#### 5.2.1. Forced Swim Test (FST)

Three-way ANOVA revealed a main effect of probiotic treatment on immobility duration ( $F_{(1, 94)} = 7.87, p < 0.01, \eta_p^2 = 0.08$ ) and a main effect of sex on climbing behavior ( $F_{(1, 94)} = 11.90, p = 0.001, \eta_p^2 = 0.12$ ). The ANOVA also found significant probiotic  $\times$  LPS treatment interaction on immobility duration ( $F_{(1, 94)} = 5.88, p < 0.05, \eta_p^2 = 0.06$ ) and a significant sex  $\times$  LPS treatment interaction on swimming duration ( $F_{(1, 94)} = 4.49, p < 0.05, \eta_p^2 = 0.05$ ).

Pairwise comparisons showed that, regardless of sex and LPS-treatment, mice exposed to sucrose control displayed greater duration of immobility than mice exposed to the *Rouxiella badensis* subsp.



**Fig. 1.** (i) Sickness scores of six-week-old (A) male and (B) female mice treated with saline or LPS and exposed to either sucrose or probiotic (*Rouxiella badensis* subsp. *acadiensis*). Data represented as mean ( $\pm$ SEM),  $n = 12$ /group (30 min – 8 h) and  $n = 8$ /group (at 12 h, 24 h and 48 h). The asterisks (\*) denote significant difference between LPS treatments (sucrose and probiotic) ( $p < 0.05$ ). (ii) Acute percent body weight change of (A) male and (B) female mice at 24 h and 48 h following saline or LPS treatment and exposed to either sucrose or probiotic treatment. Data represented as mean ( $\pm$ SEM),  $n = 8$ /group at 24 h and  $n = 12$ /group at 48 h. The asterisks (\*) denotes significant difference between treatment groups; \* $p < 0.05$ .

*acadiensis*, probiotic (MD = 23.12, SE = 8.25,  $p < 0.01$ ). Regardless of probiotic treatment, LPS-treated females displayed longer immobility duration compared to saline-treated females (MD = 23.90, SE = 11.725,  $p < 0.05$ ) (Fig. 2B). No difference was observed in males. Moreover, LPS-treated females exposed to sucrose control displayed longer immobility duration than LPS-treated females exposed to *Rouxiella badensis* subsp. *acadiensis* probiotic (MD = 65.37, SE = 16.40,  $p < 0.001$ ). Saline-treated males showed shorter swimming duration than females' counterparts (MD = -37.37, SE = 13.22,  $p < 0.01$ ). Saline-treated females showed longer swimming duration than LPS-treated females (MD = 32.52, SE = 13.22,  $p < 0.05$ ). However, LPS-treated females exposed to *Rouxiella badensis* subsp. *acadiensis*, probiotic displayed longer swimming duration than females-LPS-treated females exposed to sucrose control (MD = 48.02, SE = 18.50,  $p < 0.05$ ). Lastly, regardless of LPS and probiotic treatments, males displayed longer climbing duration than females (MD = 16.44, SE = 4.76,  $p < 0.01$ ) (Fig. 2).

### 5.2.2. Tail suspension test (TST)

Three-way ANOVA found main effect of sex ( $F_{(1, 95)} = 4.00$ ,  $p < 0.05$ ,  $\eta_p^2 = 0.04$ ) and probiotic treatment ( $F_{(1, 95)} = 5.73$ ,  $p < 0.05$ ,  $\eta_p^2 = 0.06$ ) on the duration of immobility in the TST (Fig. 3).

Pairwise comparisons showed that, regardless of probiotic and LPS treatments, females displayed longer immobility duration than males (MD = 17.11, SE = 8.55,  $p < 0.05$ ). Moreover, regardless of sex and LPS-treatment, mice exposed to *Rouxiella badensis* subsp. *acadiensis* displayed shorter immobility duration than mice exposed to the control (MD = -20.49, SE = 8.55,  $p < 0.05$ ). LPS-treated females exposed to sucrose displayed greater immobility duration than saline-treated counterparts (MD = 37.54, SE = 17.11,  $p < 0.05$ ). However, there was no significant difference in immobility duration between LPS-treated females exposed

to the probiotic and saline-treated counterparts. LPS-sucrose mice had more immobility than their LPS-probiotic counterparts (MD = 32.8, SE = 12.1,  $p < 0.01$ ) (Fig. 3A). Finally, LPS-treated females exposed to probiotic displayed significantly shorter immobility duration than LPS-treated females exposed to sucrose control (MD = -55.02, SE = 17.11,  $p < 0.01$ ) (Fig. 3A).

### 5.3. Pubertal probiotic treatment reduced anxiety-like behaviour

#### 5.3.1. Open field test (OFT)

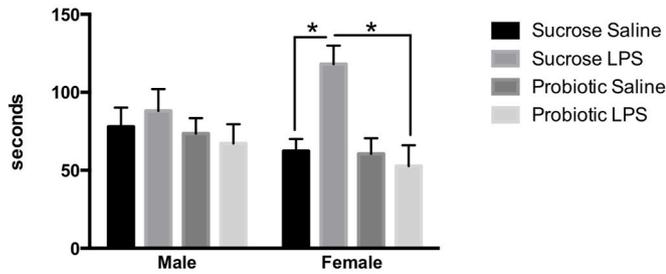
Three-way ANOVA revealed a main effect of probiotic treatment on time spent in the inner zone ( $F_{(1, 94)} = 4.87$ ,  $p < 0.05$ ,  $\eta_p^2 = 0.05$ ). Pairwise comparison showed that regardless of sex and LPS treatment, mice exposed to *Rouxiella badensis* subsp. *acadiensis* probiotic spent more time in the inner zone than mice treated with sucrose control (MD = 1.87, SE = 0.85,  $p < 0.05$ ) (Fig. 4A). Moreover, LPS-treated females exposed to probiotic spent more time in the inner zone than LPS-treated females exposed to sucrose control (MD = 3.57, SE = 1.69,  $p < 0.05$ ) (Fig. 4A). Saline-treated males exposed to *Rouxiella badensis* subsp. *acadiensis* probiotic displayed shorter latency to first enter the inner zone than their sucrose counterpart group (MD = -41.0, SE = 20.5,  $p < 0.05$ ) (Fig. 4B).

#### 5.3.2. Elevated plus maze (EPM)

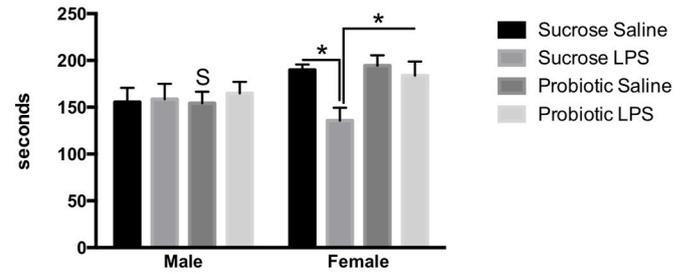
Three-way ANOVA revealed a main effect of sex on time spent in the closed arms ( $F_{(1, 94)} = 4.50$ ,  $p < 0.05$ ,  $\eta_p^2 = 0.061$ ) and a significant sex x LPS treatment interaction on travel velocity ( $F_{(1, 94)} = 4.50$ ,  $p < 0.05$ ,  $\eta_p^2 = 0.05$ ). Pairwise comparisons showed that, regardless of LPS and probiotic treatments, males spent significantly more time in the closed arms than females (MD = 13.94, SE = 5.87,  $p < 0.05$ ). Moreover, LPS-treated

### Forced Swimming Test (FST)

#### A. Immobility duration



#### B. Swimming duration



#### C. Escape duration

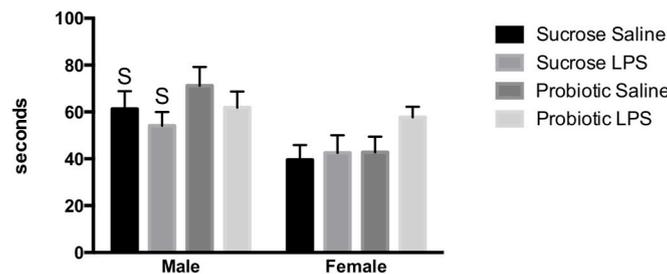
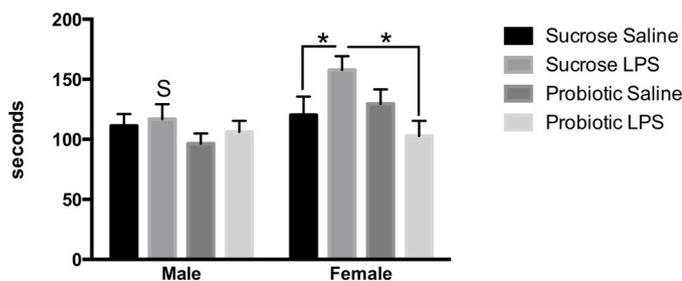


Fig. 2. Duration of immobility (A), swimming (B) or escape (C) in seconds (sec) during the forced swimming test in adult male and female mice previously treated with saline or LPS at 6 weeks of age and exposed to either sucrose or to *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) treatment during puberty. Data represented as mean ( $\pm$ SEM),  $n = 12$ /group. The asterisks (\*) indicates significant difference between treatment groups ( $p < 0.05$ ); S indicates significant difference between males and females ( $p < 0.05$ ).

### Tail Suspension Test (TST)

#### A. Immobility duration



#### B. Mobility duration

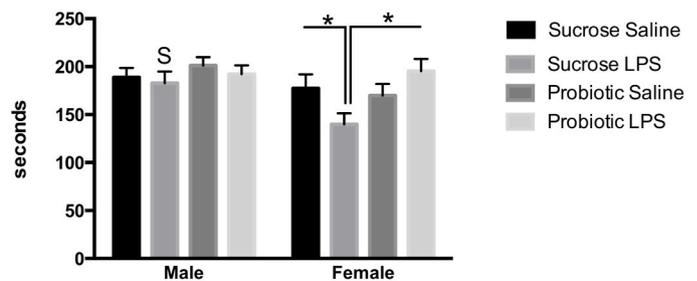


Fig. 3. Duration of immobility (A) and mobility (B) in seconds (sec) during the tail suspension test in adult male and female mice previously treated with saline or LPS and exposed to either sucrose or to *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) treatment during puberty. Data represented as mean ( $\pm$ SEM); The asterisks (\*) indicates significant difference between treatment groups ( $p < 0.05$ ); S indicates significant difference ( $p < 0.05$ ) between males and females;  $n = 12$ /group.

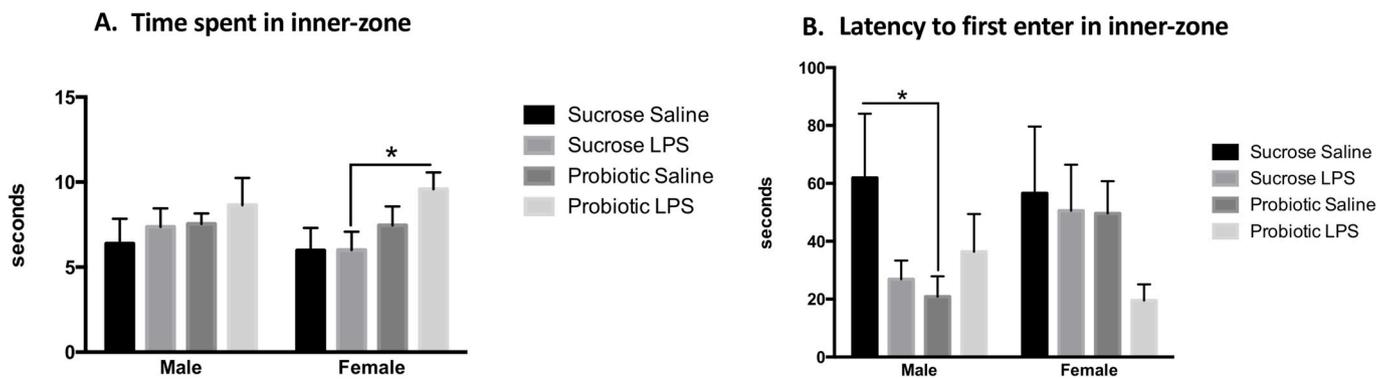
female mice displayed reduced travel velocity compared to saline-treated counterparts ( $MD = 0.715$ ,  $SE = 0.38$ ,  $p < 0.05$ ) (Fig. 5C). LPS-treated male mice exposed to the probiotic spent less time in the center zone than their saline-treated counterparts ( $MD = -25.3$ ,  $SE = 11.9$ ,  $p < 0.05$ ) (Fig. 5B).

5.4. Pubertal probiotic treatment mitigated LPS-induced changes in *5HT1A* receptors expression in specific regions cornu-ammonis (CA1) and (CA3) in the hippocampus and the dorsal raphe nuclei in a sex-specific manner

#### 5.4.1. *5HT1A* Rc expression in the DRN

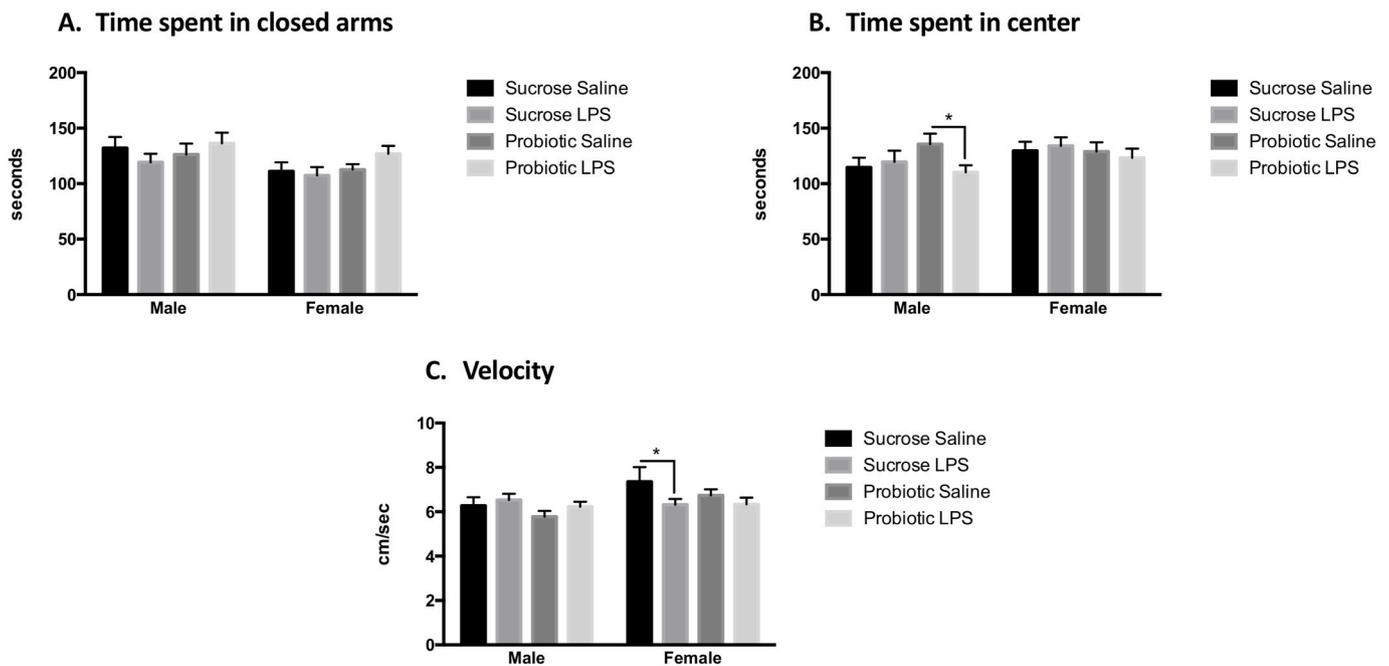
Statistical analyses revealed a main effect of probiotic treatment ( $F(1,77) = 14.51$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.17$ ) and a significant probiotic  $\times$  LPS interaction ( $F(1,77) = 6.15$ ,  $p < 0.05$ ,  $\eta_p^2 = 0.08$ ) on *5HT1A* Rc expression in the DRN. Pairwise comparisons showed that, regardless of sex and LPS

### Open Field Test (OFT)



**Fig. 4.** Duration of time (sec) spent in inner zone (A) and latency to first enter inner zone (B) during the open field test in adult male and female mice previously treated with saline or LPS and exposed to either sucrose or to *Rouxiella badensis* subsp. *acadiensis* (*Canan SV-53*) treatment during puberty. Data represented as mean ( $\pm$ SEM); The asterisks (\*) indicates significant difference between treatment groups ( $p < 0.05$ ); S indicates significant difference ( $p < 0.05$ ) between males and females;  $n = 12$ /group.

### Elevated Plus Maze (EPM)



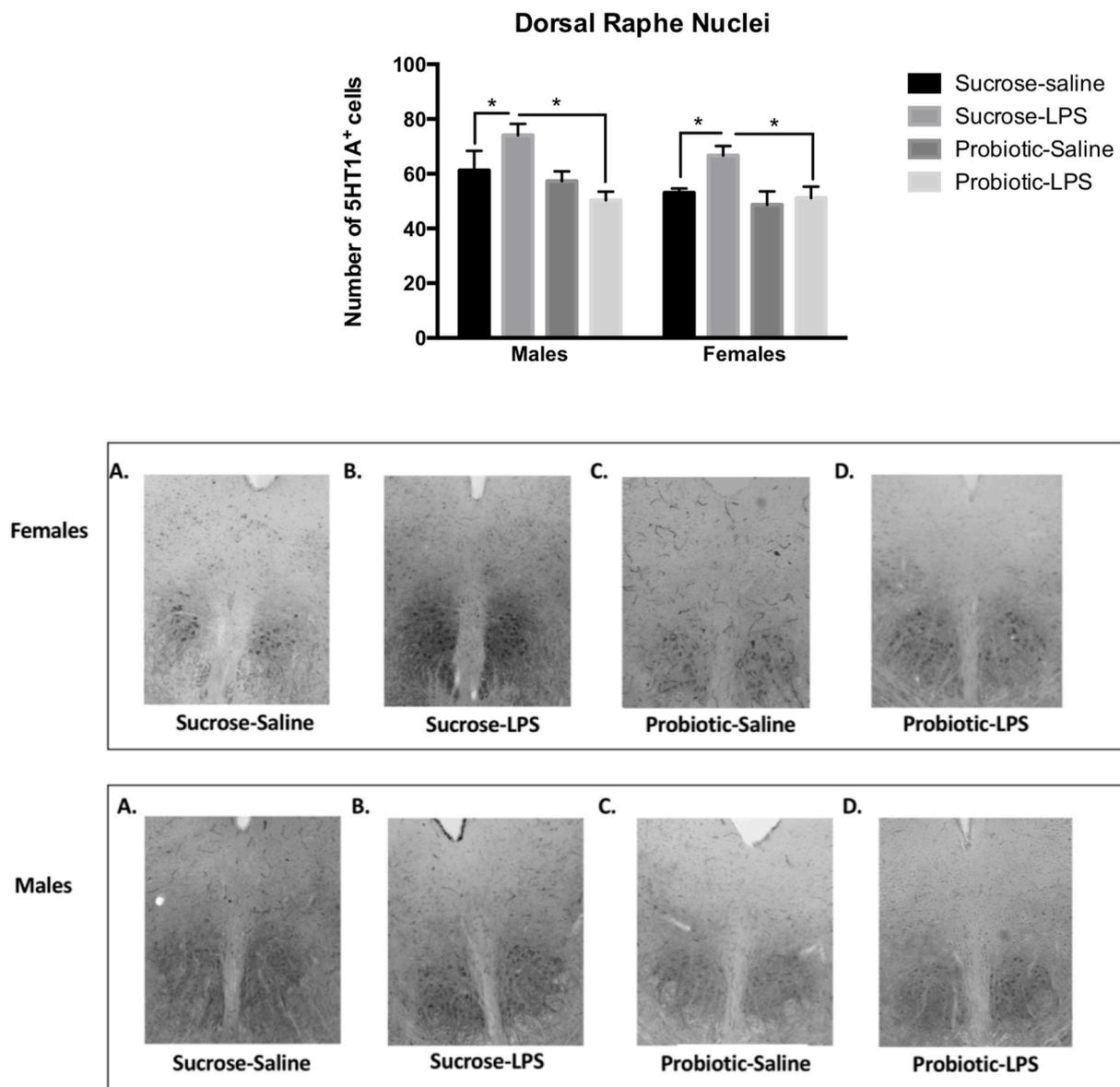
**Fig. 5.** Duration of time (sec) spent in closed arms (A) and centre (B), and velocity (cm/sec) (C) during the elevated plus maze test in adult male and female mice previously treated with saline or LPS and exposed to either sucrose or to *Rouxiella badensis* subsp. *acadiensis* (*Canan SV-53*) treatment during puberty. Data represented as mean ( $\pm$ SEM),  $n = 12$ /group; The asterisks (\*) indicates significant difference between treatment groups ( $p < 0.05$ ); S indicates significant difference ( $p < 0.05$ ) between males and females.

treatment, mice exposed to probiotics has less 5HT1A Rc expression than mice exposed to sucrose control (MD = -11.89, SE = 3.12,  $p < 0.05$ ). Moreover, regardless of sex, LPS treatment increase 5HT1A expression in the DRN in mice exposed to sucrose control (MD = 13.23, SE = 4.49,  $p < 0.01$ ). However, LPS treatment failed to alter 5HT1A expression in the DRN in mice exposed to the *Rouxiella badensis* subsp. *acadiensis* probiotic (Fig. 6).

#### 5.4.2. 5HT1A Rc expression in CA1 and CA3 of the hippocampus

Three way ANOVA demonstrated significant probiotic  $\times$  LPS ( $F(1,75) = 5.95$ ,  $p < 0.05$ ,  $\eta_p^2 = 0.08$ ) and sex  $\times$  probiotic  $\times$  LPS ( $F(1,75) =$

6.56,  $p < 0.05$ ,  $\eta_p^2 = 0.09$ ) interactions on 5HT1A Rc expression in CA1 region, as well as a significant sex  $\times$  probiotic interaction in the CA3 region ( $F(1,75) = 5.43$ ,  $p < 0.05$ ,  $\eta_p^2 = 0.07$ ). Pairwise comparison showed that, regardless of sex, LPS treatment decreased 5HT1A Rc expression in the CA1 region of the hippocampus in mice exposed to the sucrose control (MD = 16.87, SE = 7.05,  $p < 0.05$ ), but not in mice exposed to the *Rouxiella badensis* subsp. *acadiensis* probiotic. However, LPS treatment induced a greater decrease in 5HT1A Rc expression in the CA1 region in males exposed to sucrose than in female counterparts (MD = 28.18, SE = 9.78,  $p < 0.01$ ). In addition, regardless of LPS treatment, females exposed to the probiotic displayed significantly less



**Fig. 6.** Mean ( $\pm$ SEM) expression of 5HT1A in the DRN of mice previously treated with saline or LPS and exposed to either sucrose or *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) treatment during puberty (A). The asterisks (\*) indicates significant difference between treatment groups ( $p < 0.05$ ). Representative photomicrographs of 5HT1A expression in the DRN of adult female and male mice (B): A. Sucrose-Saline, B. Sucrose-LPS, C. Probiotic-Saline, D. Probiotic-LPS.

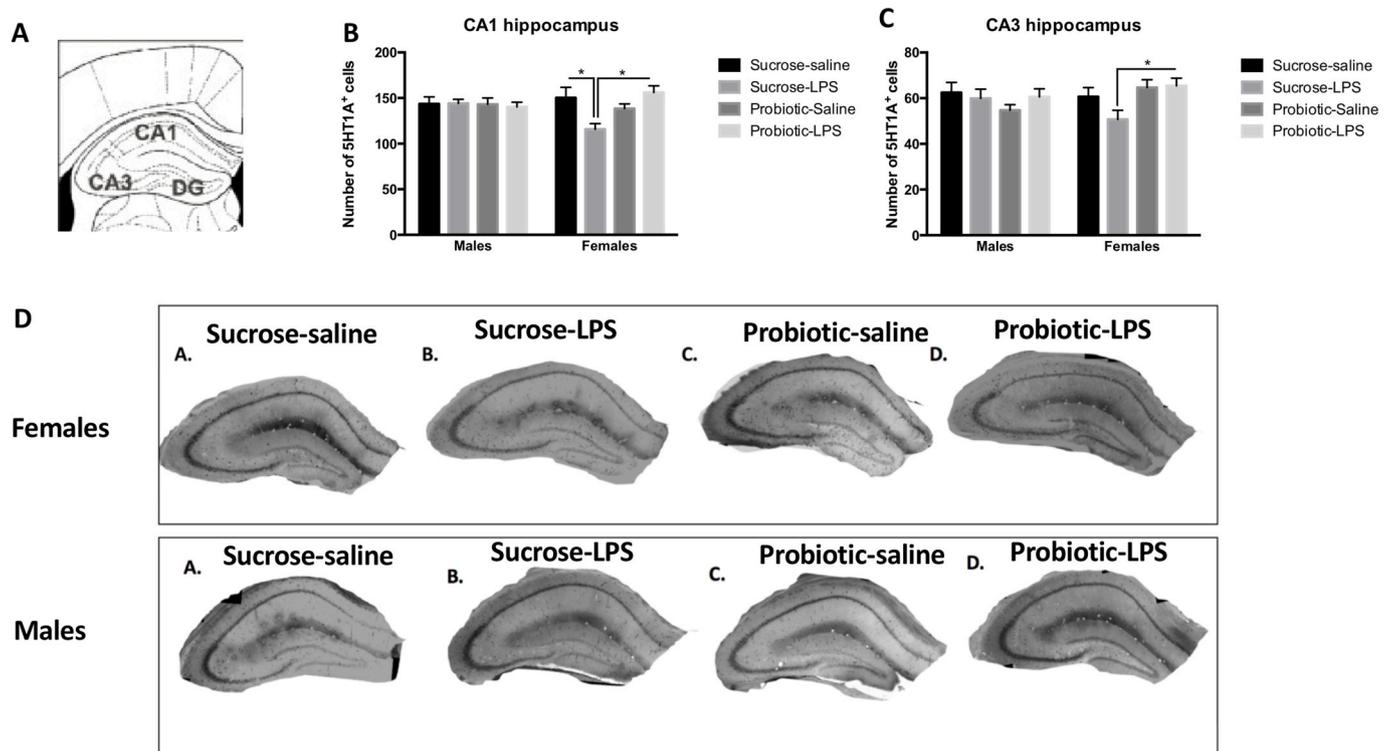
5HT1A expression in the CA 3 region compared to females exposed to sucrose control (MD = 9.17, SE = 3.94,  $p < 0.05$ ). This difference did not extend to the males (Fig. 7).

## 6. Discussion

The adolescent brain undergoes significant neuronal reorganization and remodeling of neuronal circuits that are associated with heightened vulnerability to stressors and immune challenges [2,55]. Exposure to stress or inflammation during this critical developmental period can affect brain functioning and lead to enduring changes in behaviors and adverse mental health outcomes later in life [3,54,56]. Recent findings from our laboratory showed that modulation of the gut microbiota using probiotics during adolescence can mitigate LPS-induced depression-like behaviour in females and anxiety-like behaviours in males [7,29]. Although LPS treatment during puberty is known to induce enduring depression-like and anxiety-like behaviors in adulthood, the neural mechanisms underlying these effects remain unclear. There is also limited understanding of the influence of probiotic consumption on the

developing pubertal brain. Thus, we investigated the effects of *Rouxiella badensis* subsp. *acadiensis* consumption during adolescence on LPS-induced behavioral changes in pubertal male and female mice. Given the key role of 5HT1A receptors in the pathogenesis of anxiety and depression, we investigated the effect of LPS as well as *Rouxiella badensis* subsp. *acadiensis* on the expression of these receptors in specific brain areas. We found that *Rouxiella badensis* subsp. *acadiensis* reduced LPS-induced sickness behaviours in a time dependent manner and mitigated LPS-induced depression-like behavior in an enduring manner. We also found that it mitigated LPS-induced changes in 5HT1A receptors expression in the hippocampus and in the DRN in a sex-specific manner.

All mice displayed sickness behaviours within 2 h following LPS injection with males showing more prolonged sickness behavior compared to female mice, which is consistent with previous work from our laboratory [7,54]. *Rouxiella badensis* subsp. *acadiensis* also decreased LPS-induced sickness behavior in female mice for up to 24 h following exposure to the immune challenge, while in males the improvement started 6 h after the injection. These results support our hypotheses and are in line with previous studies showing reduced sickness behavior in



**Fig. 7.** A representative schema of the hippocampal areas of interest (A), Mean ( $\pm$ SEM) expression of 5HT1A in the CA1 (B) and CA3 (C) regions of the hippocampus of adult males and females previously treated with saline or LPS at 6 weeks of age and exposed to either sucrose or to *Rouxiella badensis* subsp. *acadiensis* (*Canan SV-53*) treatment during puberty. The asterisks (\*) indicates significant difference between treatment groups ( $p < 0.05$ ). Representative photomicrographs of 5HT1A expression in the hippocampi of adult female and male mice (D): A. Sucrose-Saline, B. Sucrose-LPS, C. Probiotic-Saline, d. Probiotic-LPS.

mice following probiotic consumption, with the exception of males displaying limited improvement in sickness scores [7,57]. Increased sickness behavior in LPS-treated mice is associated with the rise of pro-inflammatory cytokines production in the blood, including IL-6, TNF $\alpha$ , IL-1 $\beta$ , IFN $\gamma$  and IL-12 [7,54]. Probiotics consumption has been shown to prevent LPS-induced production of pro-inflammatory cytokines [7,58] which may explain the reduction in sickness behaviors. The greater sickness behavior observed in probiotic-LPS treated males, compared to female counterparts, at 2 h, 4 h and 24 h post-LPS treatment, suggests a sex-dependent immune response to the bacterium and probably a stronger pro-inflammatory cytokines production in males.

We also found that LPS treatment induced weight loss in males and females at 24 h and 48 h following LPS treatment and persisted until 1 week following LPS treatment. These results are in line with previous findings [29,46] that showed weight loss at 24 h and 48 h. Although treatment with *Rouxiella badensis* subsp. *acadiensis* failed to mitigate LPS-induced weight loss at 24 h and at 48 h, it significantly reduced LPS-induced weight loss 1 week following treatment in both sexes. Moreover, probiotic treatment induced weight loss in saline-treated females at 48 h following injection which could be due to changes in gut microbiota diversity and composition affecting metabolism. Future studies should analyze changes in gut microbial composition following probiotic consumption. Nevertheless, *Rouxiella badensis* subsp. *acadiensis* was effective in reducing LPS-induced weight loss in females at 7 weeks old while no significant change in body weight was observed in male mice at this age.

Furthermore, LPS-injection at 6 weeks of age induced depression-like behaviors in adult female CD1 mice, which replicate our previous findings [7,29] and support the fact that pubertal exposure to an immune challenge alters behaviors in a sex-specific manner by affecting distinct pathways. Pubertal treatment with *Rouxiella badensis* subsp. *acadiensis* mitigated LPS-induced depression-like behaviour in females by eliminating differences in immobility duration (in FST and TST tests)

between immune- and non-immune challenged female mice. These results propose that pubertal use of probiotic prevented stress-induced depression-like behaviour in females which is coherent with previous literature related to beneficial effects of probiotic consumption on emotional behaviors such as anxiety- and depression-like behaviours [7, 29,30]. Our findings highlight the protective enduring effects of probiotics use during the pubertal period. Different mechanisms can be at origin of these behavioral changes. For example, a major role of the immune system and neuroinflammation has been associated with the development of major depression and anxiety [59,60]. Thus, inflammation induced by pubertal LPS treatment can cause enduring behavioral alterations. Other mechanisms that might be involved in the pathogenesis include alteration of the microbiota composition, transient increase in the blood brain barrier permeability and induction of central inflammation [7,29]. Protective effect of pubertal probiotic use can involve a reduction in the inflammatory reaction, a modulation of the intestinal microbiota structure, modifications of microbial metabolites levels and improvement of the integrity of the intestinal barrier [58]. Previous findings revealed the ability of *Rouxiella badensis* subsp. *acadiensis* to modulate the intestinal homeostasis and immune response by increasing the number of IgA positive cells in the small intestine and modulating inflammatory cytokine production. Furthermore, the bacterium has been shown to protect the integrity of the intestinal barrier [39].

LPS treatment at 6 weeks of age did not induce anxiety-like behaviors in males. These findings failed to replicate our previous results that LPS treatment induces enduring anxiety-like behaviors in males [7,29]. However, the absence of anxiety-like behaviors in the current study could be due to chronic stress induced by daily handling and gavage. The discrepancy between our observations and those of Murray et al. (2019) can be further explained by sex-specific effects of chronic adolescent stress on behaviors [61]. More specifically, chronic social stress (isolation, and change of cage partner) during adolescence was

found to increase anxiety-like behaviour in both sexes during adulthood, which can further explain why no difference in anxiety behaviour was observed between LPS- and saline-treated groups [62]. McCormick and colleagues have found increased anxiety-like behaviour in older males due to a greater increase in corticosterone concentrations in adult males compared to adolescent males after confinement to the open arm of the EPM [62]. These results could explain the increase of anxiety-like behavior in saline-treated males exposed to chronic stress (gavage) and the absence of a significant difference in anxiety-like behaviors between saline- and LPS-treated males. Additionally, we have observed that saline-treated mice exposed to probiotic showed no change in anxiety-like behaviors which revealed that our probiotic does not have anxiogenic effects. Moreover, the effects of pubertal immune challenge were observed several weeks after LPS exposure, indicating that sex-specific developmental trajectories and vulnerability to mood disorders may be shaped by immune system activation during critical periods of development, like puberty. Our mouse model and probiotic effects indicate that puberty can be another opportunity to shape brain development either positively or negatively.

Additionally, pubertal LPS treatment increased the expression of 5HT1A receptors in the DRN of both males and females, leading to reduced serotonin release in the projection areas and increased depression-like behaviors. Interestingly, probiotic exposure mitigated the LPS-induced increase in 5HT1A receptor expression in both sexes. Moreover, LPS-treated females exposed to probiotic had lower 5HT1A receptor expression in the DRN compared to LPS-treated females exposed to the control. Thus, *Rouxiella badensis* subsp. *acadiensis* could act as an anti-depressant by decreasing serotonin reuptake in the DRN and increasing serotonin release in projection areas.

LPS treatment also reduced 5HT1A receptor expression in the CA1 region of the hippocampus in females only, highlighting the enduring sex-specific effects of pubertal LPS challenge on 5HT1A receptor expression in this region. *Rouxiella badensis* subsp. *acadiensis* blocked the effects of LPS treatment on 5HT1A expression in the CA1 region and increased 5HT1A receptor expression in the CA1 and CA3 regions in LPS-treated females. These results demonstrate its ability to increase the expression of postsynaptic 5HT1A receptors in specific hippocampal regions. Postsynaptic 5HT1A receptors are found in limbic structures and their activation contribute to a decline in the firing rate of postsynaptic cells, resulting in antidepressant effects [63]. A reduction in postsynaptic 5HT1A receptors has been found in the hippocampi of depressed individuals and in animal models of depression [17,64,65]. Antidepressant treatment often induce the activation of these receptors [14]. These results validate the long-term depressive outcome observed upon pubertal LPS treatment in female mice and the ability of pubertal probiotic use to alleviate the negative consequences of an immune-challenge, by altering the expression of 5HT1A receptors in the hippocampus in a sex-dependent manner.

The absence of depression-like behaviors in males can be due to the fact that LPS treatment did not change the number of 5HT1A postsynaptic receptors in the CA1 and CA3 regions.

## 7. Conclusion

The current study underscores the key role of puberty as a critical phase during neurodevelopment with heightened level of vulnerability to immune challenges. The results reveal that exposure to an immune challenge during puberty changes 5HT1A receptor expression in different brain regions and causes enduring depression-like behavior in females. Furthermore, our study demonstrates that probiotics like *Rouxiella badensis* subsp. *acadiensis* can mitigate the long-term neural and behavioral alterations induced by pubertal LPS. Further investigations remain necessary to fully elucidate the impact of probiotics during adolescence in the prevention of stress-induced mood disorders.

## Funding

This publication was funded by an NSERC Collaborative Research and Development Grant (532223-18) to C.M., Admission Scholarship by Faculty of Graduate and Postgraduate Studies at the University of Ottawa to N.Y. and Nutrition and Mental Health Doctoral Scholarship by the Nutrition and Mental Health initiative at the Faculty of Health Sciences at the University of Ottawa to N.Y.

## Declaration of competing interest

We have no conflict to declare.

## Acknowledgment

We would like to thank Jacky Liang for all his technical help and support. We are grateful for the assistance provided by Tama Davis and the ACVS staff at the University of Ottawa, Kevin Smith, Briallen Taylor, Harshita Seal and Taha Mohamed for their help.

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