EFFECTS OF PROBIOTIC ON RESPONSES TO STRESS:

SYSTEMIC MODULATION OF MICROBIOTA-GUT-BRAIN AXIS

By:

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TITLE: Effects of probiotic on responses to stress: systemic modulation of microbiota-gut-brain axis

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LAY ABSTRACT

Excessive exposure or dysregulated responses to stress in human and animals induces behavioral changes and the development of mood disorders. The Microbiota-gut-brain axis plays an important role in maintenance of homeostasis. However, crosstalk between the different components of microbiota-gut-brain axis, and how specific microbes can modulate these interactions, remains unclear. Thus, we sought to understand the mechanism of inter-systemic communication linking a specific gut microbe to changes in stress response and behavior. We observed immunoregulation by regulatory T cells were essential in *Lactobacillus rhamnosus* JB-1 induced anxiolytic and antidepressant-like effects. We also found the integrity of vagus nerve was necessary for JB-1 induced promotion of regulatory T cells and decrease in microglial activation and attenuation of hypothalamic-pituitary-adrenal axis that are associated with the anxiolytic effects of the bacteria. We also identified that the temporal relationship between exposures to stress and the bacteria is important as ingestion of JB-1 directly after chronic social defeat lead to persistence of fear memory and social deficits. This work will help us to understand mechanisms underlying the microbiota-gut-brain axis, which may allow for the development of novel microbe based therapeutic intervention against mood disorders.

ABSTRACT

Bacteria, especially symbiotic species in the gastrointestinal tract, have lived with human for long time and are involved in many aspects of host physiology. There is growing evidence that microbiota-gut-brain axis plays an important role in modulating the response to stress in both human and animals. Alterations in the gut microbiota can change the central nervous system function through effects on the endocrine, immune and nervous systems. Recent studies suggest that probiotic treatment may help to maintain resistance against the detrimental effects of stress though the microbiota-gut-brain axis. However, how potentially beneficial bacteria interact with specific immune and neural components, to mediate beneficial effects on behavior remain unclear. Using chronic social defeat stress, a model often used in post-traumatic stress disorder research, we found that administration of *Lactobacillus rhamnosus* JB-1 beginning 48 hours following chronic social defeat led to persistence of fear memory and social deficits. These effects were associated with changes in gene expression related to emotion and memory in the hippocampus. This was in contrast to previous studies showing that probiotic intervention during social defeat prevents stress induced deficits in social behavior. This indicates that timing of *L. rhamnosus* treatment in relation to stress exposure has important implications for effects of the bacteria on behavior. In relation to the mechanism of action of *L. rhamnosus* on behavior, we demonstrate through depletion and adoptive transfer experiments that $CD4^+CD25^+$ T cells in

mice treated with JB-1 were necessary and sufficient for JB-1 induced anxiolytic and antidepressant-like effects. Evidence also suggested that Ly6C^{hi} monocytes may be a downstream target inhibited by Tregs involved in the behavioral effects of the bacteria. We observed that JB-1 could also reduce the number of activated microglia in the hippocampus, and attenuate hypothalamic-pituitary-adrenal axis reactivity with the integrity of vagus nerve. Crucially we demonstrated that JB-1 induced promotion of peripheral Tregs, reduction in microglia activation in the hippocampus, and attenuation of HPA axis reactivity, were all inhibited following vagotomy indicating that vagus nerve integrity is required to maintain immune and endocrine linkages from gut microbes to the brain. These studies demonstrate prerequisites for beneficial probiotic effects on stress related behaviours including a specific time window in relation to stress exposure, the activation of regulatory immune cells, and undisrupted vagal nerve signalling. These findings highlight the inter-systemic communication of the microbiota-gut-brain axis in the stress response, and might help to unveil more therapeutic opportunities in relation to stress-related mood disorders.

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"They who know the truth are not equal to those who love it, and they who love it are not equal to those who delight in it." -- Confucius, Analects, 475-221 BC

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LIST OF ABBREVIATIONS AND SYMBOLS

AVPR	Arginine vasopressin receptor
BDNF	Brain-derived neurotrophic factor
CCL-2	C–C motif chemokine ligand 2
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CNS	Central nervous system
CSD	Chronic social defeat
CRF/CRH	Corticotropin-releasing factor/hormone
CRHR	Corticotropin-releasing hormone receptor
DAPI	4′,6-diamidino-2-phenylindole
DCs	Dendritic cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus maze
Foxp3	Fork-head box P3
FSC	forward scatter
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GF	germ-free
GR	Glucocorticoid receptor

HPA Axis	Hypothalamic-pituitary-adrenal axis
Iba-1	Ionized calcium-binding adaptor molecule 1
IFN	Interferon
IL	Interleukin
JB-1	*Lactobacillus rhamnosus* (JB-1)
LDT	Light-dark test
Ly6C	Lymphocyte antigen 6C
MDD	Major depressive disorder
MHC	Major Histocompatibility complex
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
OFT	Open field test
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
PTSD	Post-traumatic stress disorder
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SEM	Standard error of mean
SSC	Side scatter
SSRI	Selective serotonin reuptake inhibitor
Th1/Th2 Cell	T helper cell 1/2

TNF	Tumor necrosis factor
Tregs	Regulatory T Cells
TST	Tail suspension test
Vx	Vagotomy

DELARATION OF ACADEMIC ACHIEVEMENT

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The author's contributions associated with each study are presented sequentially. Chapter 3: chronic social defeat, probiotic/sertraline administration, behavioral tests, tissue collection and process, PCR, flow cytometry, data analysis and interpretation, preparation and writing of the associated manuscript. Chapter 4: depletion, cell sorting and adoptive transfer of regulatory T cell, probiotic administration, behavioral tests, tissue collection and process, flow cytometry of splenocytes, data analysis and interpretation, preparation and writing of the associated manuscript. Chapter 5: vagotomy/sham surgery and post-operative care; probiotic administration, behavioral tests, acute restraint stress, tissue collection and process, immunohistochemistry of brain sections, PCR of hippocampus, ELISA of plasma cortisol, flow cytometry, data analysis and interpretation, preparation and writing of the associated manuscript.

CHAPTER 1: INTRODUCTION

1.1. Stress, PTSD and animal models.

Stress, first described in the field of medical research by Dr. Hans Selye in 1936, is a conscious or unconscious psychological feeling from specific physical or chemical risk factors (cold, virus, toxin, etc.), or nonspecific 'stressors' (emotion, neuroendocrine, gastrointestinal alterations, etc.) (Szabo, Tache, & Somogyi, 2012). Psychological stress was defined by Lazarus et al. as ‘a particular relationship between the person and the environment that is appraised by the person as taxing or exceeding his or her resources and endangering his or her well-being’ (Folkman and Lazarus, 1984). The stress response is a normal function of all animals, and high-level adaptation to stress is a key survival advantage. Once an animal encounters stress, the sympathetic nervous system is activated, and catecholamines drives the animal to a 'fight-or-flight' state. However, if the stress is prolonged, the hypothalamic-pituitary-adrenal axis will be more involved to limit the duration and damage of initial stress response as a strategy to redistribute energy (Radley et al., 2011). This complicated adaptive system helps the body to react accurately and immediately to stressors but also maintain homeostasis. However, if the stressor is too strong, or prolonged to become chronic stress, it can lead to loss of integrity of the adaptive system and cause neurological and psychological damage. In humans, the consequences of loss of the adaptive response can lead to disruption of CNS function and to psychiatric disorders.

Posttraumatic stress disorder (PTSD) is a debilitating disorder with major social and economic impacts (Greenberg, Brooks, & Dunn, 2015; Pace & Heim, 2011). PTSD develops following exposure to traumatic events and is characterized by symptoms including hyper-arousal and changes in mood and cognition. In the latest version of Diagnostic and Statistical Manual of Mental Disorders, DSM-V, PTSD classified as a "Trauma- and Stressor-Related Disorders" (American Psychiatry Association, 2013). The main characteristic of PTSD is that the patient must have experienced a period of extreme stress at least once, which causes failure of adaptation and abnormal reaction to triggers with a close relationship to the initial stressor (He, Glas, & Veldkamp, 2014).

Amongst the general population in North America the lifetime rate of PTSD is estimated at 8-9 %, with one-year prevalence rates of approximately 3 to 5 % (Goldstein et al., 2016; Kessler, Chiu, Demler, & Walters, 2005; Van Ameringen, Mancini, Patterson, & Boyle, 2008). A much higher prevalence is observed in combat veterans or individuals exposed to war, with a recent study finding that around 19% of combat exposed soldiers screened positive for PTSD (Hoge, Riviere, Wilk, Herrell, & Weathers, 2014). It is estimated by the US government that the economic cost of PTSD is more than 3 billion dollars per year (Kessler, 2000). Another important issue is that the majority of people with this disorder do not seek professional help. It has been demonstrated that around 70% of people who suffer with PTSD are not seeking any professional help for their condition (Greenberg et al., 2015). Another epidemiologic study indicated that more than 90

percent of people, during their life time, would encounter at least one severe stress event, but only a small proportion of this population will have difficulties in adaptation, with a recovery period longer than a month (one month is the least diagnostic period for PTSD) (Ogle, Rubin, Berntsen, & Siegler, 2013). Thus, the development of PTSD depends not only the exposure to stress, but also other important inter-individual factors. A twin-pair study of subjects in the military demonstrated that having a monozygotic twin with PTSD is associated with higher risk of developing PTSD (True et al., 1993). Some genetic factors have also been found to be correlated with PTSD (Roth, Zoladz, Sweatt, & Diamond, 2011; Sarro, Sullivan, & Barr, 2014; Smoller, 2016). Also, research has demonstrated that PTSD is associated with comorbidities, with patients often also having anxiety, depression and substance (drugs, alcohol) abuse (Back et al., 2014; Simmons & Suárez, 2016; Spinhoven, Penninx, van Hemert, de Rooij, & Elzinga, 2014).

Certain animal models based on stress exposure are considered good PTSD models due to their etiological, face and construct validities (Daskalakis, Yehuda, & Diamond, 2013). For example, a single foot electric shock can lead to fearfulness, social withdrawal and depressive-like behavior (Siegmund & Wotjak, 2007). Models based on early-life stress, like chronic early life stress (using a limited nesting area for breeding), also leads to enduring disruption of the HPA axis, neuronal dysfunction in hippocampus, and reduced adult neurogenesis (Naninck et al., 2015; Rice, Sandman, Lenjavi, & Baram, 2008). Repeated restraint stress, a simple animal model to restrict the movement of animals by constraining them in

cylinders or wire mesh, is demonstrated as an effective method to induce anxious behaviors (Chotiwat & Harris, 2006). Chronic social defeat, as a classic model of learned helplessness in rodents, is demonstrated to be particularly effective in establishing abnormal behaviors and cognitive impairment relevant to PTSD (Reader et al., 2015; Willis & Blaney, 1978). Specifically, chronic social defeat can cause anxiety-like and depression-like behaviors, as well as altered spatial cognition. The chronic social defeat model also leads to structural changes in the brain, it can reduce expression of synaptic proteins and dendritic spine structural plasticity in the frontal association cortex (Jianhua, Wei, Xiaomei, & Shao-Hui, 2017; Shu & Xu, 2017). Previously, the chronic social defeat model has been used to test the preventive effects of probiotics on the development of stress induced behavioural and immunological changes (Bharwani, Mian, Surette, Bienenstock, & Forsythe, 2017).

Besides multiple types of stress-exposure model, it is important to note, that some specific strains of mice naturally display high levels of anxious and depressive-like behaviours at 'baseline' (in the absence of stress exposure). Thus, stress-exposure models can be divided into two categories: one is 'conditioned stress', such as chronic social defeat or electric shock exposure, which lead to pathologic behavioral changes, the other is 'unconditioned response', which usually does not need a specific period of stress introduction. Common behavior tests, such as light-dark choice task, elevated plus maze or social interaction, if being tested on specific strains of mice, can generate results which can be indices

for spontaneous, 'trait' behavioral response. In relation to trait behaviours there are many differences between C57BL/6 and BALB/c mice. For example, electric footshock induces a higher increase in dopaminergic turnover in the prefrontal cortex of BALB/c mice than in C57BL/6 mice (Herve et al., 1979). C57BL/6 mice also display higher baseline activity and exploration in a new environment through light/dark test, suggesting a low level of anxiety, compared to BALB/c. C57BL/6 also show higher sensitivity to the anxiolytic effects of treatment with diazepam than BALB/c mice (Lepicard, Joubert, Hagneau, Perez-Diaz, & Chapouthier, 2000). BALB/c mice thus may be considered as a valid model of 'trait' anxiety and depressive-like behavior, which is related to various neurotransmitters systems (GABAergic, dopaminergic, opioidergic, etc.) (Belzung & Griebel, 2001). In conclusion, there are several animal models that can be used to study mood and anxiety related disorders, sometimes we need both trait and conditioned experiments.

1.2 Current treatment of PTSD.

PTSD, though regarded as a psychiatric disorder, is demonstrated to have a complicated pathophysiological mechanism with many systemic changes involved. The three main adaptive systems, endocrine, nervous and immune systems, have all been the focus of research related to PTSD (Pace & Heim, 2011). One characteristic of PTSD patients is a disruption of stress response systems, they generally have higher levels of norepinephrine both peripherally and centrally,

and some studies have identified a reduced level of corticosteroid secretion (Kosten, Mason, Giller, Ostroff, & Harkness, 1987; Sriram, Rodriguez-Fernandez, & Doyle III, 2012; Young & Breslau, 2004).

Current treatments of PTSD include trauma-related therapies, pharmacological treatment) and other therapies like exercise and different types of group therapy. Two most commonly applied trauma-related therapies are exposure therapy and cognitive behavioral therapy (CBT). Although early-stage recovery has been demonstrated using exposure therapy and CBT, both types of therapy are associated with some relapse of event-related symptoms like flashbacks, and also have limited effects on other symptoms like anxiety or numbness (Taylor et al., 2003). There is a study suggesting that a subgroup of patients treated with exposure therapy have a worsening of PTSD symptoms through the treatment (Tarrier, Sommerfeld, Pilgrim, & Humphreys, 1999).

Selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are first-line drugs for PTSD patients, according to the current Clinical Practice Guidelines. Several clinical studies have demonstrated that SSRIs such as fluoxetine and sertraline can cause long-term relief of specific symptoms like hyperarousal or numbing, but the rates of symptomatic improvement among distinct populations can vary (40% in civilians vs. 15% in combat-exposed subjects), in a way which suggests that patients with more stressful trauma-like events show reduced response to the treatment (van der Kolk et al., 1994). Moreover, although many studies show that SSRIs can help

to improve overall clinical scores, other studies found there were few beneficial effects of fluoxetine in PTSD patients. Berg et al, reviewed several randomized controlled trials and concluded there was insufficient evidence to support the efficacy of SSRIs on PTSD treatment (Berg et al., 2007). Thus, whether SSRIs are good choices for patients with PTSD or even particular sub-groups of patients is still unclear. However, recent clinical trials indicate that early-stage administration of SSRI (early stage was defined as the period after the exposure to trauma and before the diagnosis was made, usually 1 month) did not show any preventive effect in PTSD (Shalev et al., 2012) (Zohar et al., 2018). Thus, the overall efficacy, as well as the ideal timepoint to start administration of SSRIs for PTSD patients need further elucidation.

Other pharmacotherapies of PTSD include SSNIs, Tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), benzodiazepines and anti-adrenergic agents. Most of these drugs have low-level selectivity, and lack support from randomized controlled clinical trial.

Since encounter with stress is inevitable in life, it would be ideal that an effective early-stage intervention be found to reduce the onset of PTSD. There has been limited clinical study of such approaches. One clinical trial used hydrocortisone to treat 20 individuals after septic shock. After a 31-month follow up, 7 out of 11 people in placebo group were diagnosed, while only 1 out of 9 people in hydrocortisone group was diagnosed as PTSD, suggesting that anti-inflammatory treatment may reduce the incidence of PTSD (Schelling et al., 2001).

Moreover, propranolol, as one of the β blocker family, showed preventive effects in young people had traffic accident. 3 out of 8 people who refused to use propranolol had PTSD, 2 months after exposure to trauma, while only 1 out of 11 people in treatment group had the diagnosis of PTSD in the end (Vaiva et al., 2003). These clinical results suggest that both the HPA-axis and adrenaline system are involved in the pathogenesis of PTSD. However, clinical use of high dosage corticosteroid directly after exposure to trauma is comparatively risky, as it can cause impairment of wound healing, perioral dermatitis, or even the spread of infection (Lipworth, 1999). Psychiatric adverse effects induced by corticosteroid were also reviewed by Warrington et al, and they identified that approximately 28% of short-term corticosteroid users had psychiatric symptoms like euphoria and hypomania (Warrington & Bostwick, 2006). Thus, there is a need for a safe and effective treatment to protect people from developing PTSD following exposure to traumatic stress. Utilizing an animal model of chronic social defeat, our laboratory demonstrated that continuous administration of *L. rhamnosus* JB-1 (before and during the stress exposure) could attenuate anxiety-like behavior and deficits in social interaction caused by stress, these protective effects were correlated with a reduction in the population of activated dendritic cells and increase in IL-10^{+} regulatory T cells in spleen (Bharwani et al., 2017). These results indicate that consumption of JB-1, prior to or during exposure to social-defat, can aid in resistance to social defeat-induced stress, and this procedure is related to decreased peripheral inflammatory statues, as well as alterations in specific areas

in the central nervous system. In this thesis, an experiment focused on JB-1/SSRI treatment directly after defeat is described.

Overall, PTSD is a serious medical and social problem. Our understanding of the disorder is still in the early stages and more research, especially the exploration of linkage between immune imbalance and behavioral malfunction, is required if more effective therapeutic approaches are to be developed.

1.3 Microbiota-gut-brain axis controls stress response in animals.

Microbiota-gut-brain axis is a term defined as signaling between the gastrointestinal tract and the central nervous system, and with an emphasize on the important role played by gut microbiota (Bienenstock, Kunze, & Forsythe, 2015; Mayer, Tillisch, & Gupta, 2015; Petra et al., 2015). The main components of this axis are the nervous system, immune system, endocrine system and gut microbiota (Y. Wang & Kasper, 2014). In 2004, a seminal study by Sudo *et al.* demonstrated that germ-free BALB/c mice (with no gut microbiota) showed an exaggerated HPA axis response to restraint stress than non-germ-free mice (Sudo et al., 2004). In the years since the work of Sudo et al. there have been many breakthroughs in our understanding of the microbiota-gut-brain axis.

More than 1,000 species of bacteria make up the gut microbiota of human beings, with approximately 10^{13} microbes, which is approximately equal to the total number of cells present in the human body, and with the genome (microbiome) 150 times greater as compared to human genome (Parashar & Udayabanu, 2016;

Rajilić-Stojanović & de Vos, 2014). Classically, the gut microbiota participates in multiple physiological functions including carbohydrate metabolism, food fiber degradation and maintenance of immune system. One theory widely accepted now is that gut microbiota is also involved in regulation of behavior and pathological psychiatric changes. Dysbiosis of gut microbiota is related to depression, anxiety, autism and Alzheimer's disease (Crumeyrolle-Arias et al., 2014; Mangiola et al., 2016; Pistollato et al., 2016; Slyepchenko et al., 2017). It is obvious that gut microbiota and humans coexist in a mutualistic relationship, and studies of gut microbiota will help us understand human physiology.

Previous works of our laboratory demonstrated that one specific strain of probiotic, *Lactobacillus rhamnosus* JB-1, if given orally to BALB/c mice for 28 consecutive days, could reduce anxiety-like and depressive behaviors. JB-1 could also modulate the mRNA expression levels of $GABA_{A\alpha2}$, $GABA_{A\alpha1}$, and $GABA_{B1b}$ receptor subunits, which were found to be correlated with abnormal behaviors, mainly anxiety, in a brain area-dependent manner (Bravo et al., 2011b). Other researchers have also demonstrated similar effects of probiotics. *Lactobacillus plantarum* PS128 had psychotropic effects, including increased locomotor activity and decreased depression-like behaviors, in mice subjected to early life stress, and these behavioral changes were accompanied by lower levels of inflammatory cytokines including TNF-α and IL-6, and higher levels of IL-10 (Y.-W. Liu et al., 2016). Also, some strains of probiotics can prevent the visceral impairment induced by psychological stress. For example, restraint stress on rats causes colonic

hypersensitivity, indicated by lower level of colonic distention threshold, while 8-day oral feeding of *Lactobacillus rhamnosus* Lcr35 prevented this stress induced decrease in threshold (Darbaky et al., 2017). On the other hand, antibiotic treatment disturbs the integrity of normal gut microbiota and can lead to disruption of behavior. Our laboratory found that low-dose penicillin in late pregnancy and early postnatal life (*in utero* until weaning) led to impaired anxiety-like behavior and social behaviors, including increased aggression, while concurrent supplementation with JB-1 prevented some of these alterations (Leclercq et al., 2017). Sex of animals appears to influence the effect of antibiotic when exposure occurs in either pre- or early postnatal periods. Champagne-Jorgensen et al. demonstrated that prenatal exposure to low-dose penicillin had sex-specific effects on adult mice, for males had higher level of anxiety-like behaviors and lower regulatory T cells, while exposed females developed decreased anxiety-like behaviors, and these phenomena might be due to differentially altered microbiota composition (Champagne-Jorgensen et al., 2020). Similarly, Kayyal et al. found that postnatal administration of low-dose penicillin, 1 weak prior to weaning, led to long-term changes in social behavior in male mice only, and correlated with decreased expression of arginine vasopressin receptor (AVPR) 1a and 1b in the hippocampus (Kayyal et al., 2020), effects that could be prevented by concurrent treatment with JB-1. All the above results demonstrated that the eubiosis of the gut microbiota is necessary in maintaining the behavioral normality, and probiotic

treatment could help the subjects to resist stress induced effects on behavior potentially through hormonal, neural and immune pathways.

1.4 Inflammation in stress response.

Stress can cause systemic and regional inflammation and lead to pathologic changes in the brain. PTSD patients are demonstrated to have increased levels of inflammatory markers, and a higher co-morbidity of autoimmune diseases (Benros, 2015; Wieck, Grassi-Oliveira, do Prado, Teixeira, & Bauer, 2014). Several pro-inflammatory cytokines, such as IL-6, and IFN-γ, were demonstrated to be increased in the peripheral circulation, the regional gut-associated lymphoid system, and the nervous system. Recent studies also identified that activated mononuclear cells ($Ly6C^{hi}$ monocytes in mouse) are associated with stress induced disruption of behavior. Following psychiatric stress, the monocyte population in the circulation is increased and monocytes are recruited from the circulation across blood-brain barrier leading to neural inflammation and an associated increase in anxiety-like behaviour (Wohleb et al. 2013). Thus, the $Ly6C^{hi}$ monocytes can be considered as important communicators between the immune system and CNS.

Microglia are the major immune cells in the CNS, and account for about 5-10% of all brain cells (Frost & Schafer, 2016). In normal healthy brain, microglia usually stay in a quiescent state, with a ramified shape and short processes. Once a challenge (inflammation, injury, or stress) occurs, the morphology of microglia

starts to alter to an active type, with prolonged processes and inflammatory mediator production (Banati et al., 2000). Microglia are one of the major regulators of multiple neuroimmune activities, including production of cytokines like IL-1β and TNF-α, and neurotoxic substances like reactive oxygen species (Singhal & Baune, 2017), microglia are involved in modification of synapses and neuronal networks (i.e. neuroplasticity) (Sandvig, Augestad, Håberg, & Sandvig, 2018), and help cortex development by regulating the number of neural precursor cells (Cunningham, Martínez-Cerdeño, & Noctor, 2013). Activated microglia have been demonstrated to be correlated with several brain disorders include Alzheimer's disease (Hansen, Hanson, & Sheng, 2018), Parkinson's disease (Ho, 2019), multiple sclerosis (Luo et al., 2017), brain tumors (Gutmann & Kettenmann, 2019), and stroke (Heindl et al., 2018).

Recent studies have addressed the importance of microglia in psychiatric disorders. Torres-Platas et al. were first to demonstrate activated microglia were increased in psychiatric patients. In their research, post-mortem brain samples were collected from 24 middle-age patients who had died by suicide with associated depression. They found that the gene expression of one microglia-specific marker, ionized calcium binding adaptor molecule (Iba-1), was significantly increased in dorsal anterior cingulate cortex in depressed suicides. Moreover, monocyte chemoattractant protein-1 (MCP-1) was also increased, indicating that these patients have both enhanced microglia priming and monocyte recruitment (Torres-Platas, Cruceanu, Chen, Turecki, & Mechawar, 2014). Some studies have

also found increased microglia activity in schizophrenia patients (Bloomfield et al., 2016; Van Berckel et al., 2008). Many animal studies have focused on the relationship between stress exposure and maturation of microglia. By using brief daily maternal separation, an early-life stress model, Delpech et al. found increased density (indicated by Iba-1^{+} cells) and altered morphology of microglia in the hippocampus of 14-day old pups, and the density was reduced within the time span of 14 to 28 days, but the behavioral differences remained until 35-38 days (Delpech et al., 2016). By using 6-cycles of repeated social defeat, Wholeb et al. found enhanced Iba-1^{+} signals in specific brain regions, and the Iba-1^{+} expressing microglia also showed alterations in morphology including increased cell size and shortening of cell processes (Wohleb et al., 2011). The same group also found that mice deficient in chemokine receptors associated with monocyte trafficking (chemokine-receptor 2, CCR-2^{ko}) or fractalkine receptor knockout (CX3CR1^{ko}) had reduced macrophages recruitment, as well as microglial activation in the brain, and the mice did not show anxiety-like behavior (Wohleb et al., 2013). A recent study by Li et al. found that in a mouse PTSD model induced by electric foot-shocks, the ratio of microglia was significantly increased on the 5th day of stress, temporal patterns of microglial activation were found in the hippocampus of PTSD brains, and depletion of microglia could alleviate PTSD-associated anxiety and contextual fear (Li et al., 2021). All these results indicate that microglial activation, along with monocytes/macrophages recruitment, contribute to the development of neuroinflammation and behavioral abnormality in stress response.

Gut microbiota can influence microglia. In an Alzheimer's disease model, mice treated with *Clostridium butyricum* showed less cognitive impairment, less activated microglia in the cortex and hippocampus, and lower expression of proinflammatory cytokines including TNF-α and IL-1β (Sun et al., 2020). In other research, *L. paracasei* reduced the level of microglial activation and ROS production in the hippocampus of rats on a high fat diet (Chunchai et al., 2018).

In addition to changes in cellular immune profile, many studies have demonstrated alterations in serum inflammatory markers in association with PTSD. High level of the inflammatory cytokine IL-1β have been detected in individuals with PTSD and in animal models of the disorder (as reviewed by Waheed, Dalton, Wesemann, Ibrahim, & Himmerich, 2018). Elevated (10 fold) IL-6, as well as increased IL-6 receptor concentrations, were found in early-stage PTSD patients (Maes et al., 1999). Serum IL-6 were also able to predict development of PTSD in young people 6 months after a motor vehicle accident (Pervanidou et al., 2007). Similarly, TNF-α and IL-1β levels were increased in subjects who developed PTSD following a deadly earthquake (W. Wang et al., 2019). Moreover, pro-inflammatory cytokines were found to be elevated in the central nervous system in animal models with PTSD. For example, rats exposed to enhanced single prolonged stress were found to have increased levels of IL-6 and IL-1β in the hippocampus (Peng et al., 2013). However, it should be mentioned that contrasting results suggesting PTSD is associated with neuroimmune suppression were also reported. Bhatt et al. demonstrated that higher C-reactive protein levels were associated with lower

PTSD severity, measured by scores of the Clinician Administered PTSD Scale and through imaging of the 18-kDa translocator protein (TPSO), a microglial biomarker, by positron emission tomography (Bhatt et al., 2020). Mice exposed to unpredictable stress also showed neuroimmune suppression, which was explained by glucocorticoid receptor antagonism, and was associated with reduction of neuronal remodeling, mediated by microglia (Horchar & Wohleb, 2019). To sum up, evidence indicates that inflammatory and psychiatric pathology are tightly linked in stress response.

1.5. Immunoregulation and the microbiota-gut-brain axis.

To maintain the immune balance, immune regulation is usually initiated after the onset of inflammation. $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs), as a major anti-inflammatory cell population, were demonstrated to be significantly reduced in individuals with PTSD. Normal functions of Tregs include release of anti-inflammatory cytokines like tumor necrosis factor-β (TNF-β) and IL-10, inhibition of antigen presentation by dendritic cells while increasing their regulatory function, and prevention of co-stimulation signals such as CD28 (Vignali, Collison, & Workman, 2008). Alterations in the population and function of Tregs have been shown to be related to the onset of psychiatric disorders. Sommershof et al. found a significant reduction of Tregs in 19 individuals with war and torture related PTSD, compared to normal and trauma-exposed controls (Sommershof et al., 2009). In children with autism, a higher rate (about 73.3%) of Treg deficiency was found, and

these children usually had allergic manifestations (40%), suggesting an overall imbalance of the immune system (Mostafa, Al Shehab, & Fouad, 2010). Antidepressant therapy on 16 patients suffering from a depressive episode led to increases in the Treg population and reduced levels of IL-1β and IL-6 (Himmerich et al., 2010). In microbiota-gut-brain axis, Tregs involve in inhibition of excessive stress responses, and some probiotics are able to increase the proliferation of Tregs. Our lab previously found that 9 days oral feeding of JB-1 led to increased population of Tregs in Peyer's patches and mesenteric lymph nodes, with reduction of TNF and interferon-γ (IFN-γ), and up-regulated hemoxygenase-1 in dendritic cells, which was correlated with inhibitory immune activity (Karimi, Kandiah, Chau, Bienenstock, & Forsythe, 2012). In this thesis, two experiments discussing Tregs and microglia in stressed mice after probiotic administration were included, in order to broaden the understanding of microbiota-gut-brain axis and stress response, mainly on the aspect of immune function.

1.6 The vagus nerve plays important roles in stress responses.

The vagus nerve is one of the main linkages between intestine and brain, which could transmit the signal and modulate function bi-directionally. Anatomically, the vagus nerve leaves from medulla oblongata on two sides, passes through jugular foramen, then travels down to the neck, chest and abdomen, branching to innervate, and exert control over, almost all visceral organs. The right vagus nerve, according to the position on the cervical segment, descends posterior to the right

main bronchus and forms the posterior vagal trunk, while the left vagus nerve pass through the esophageal hiatus at the anterior position, thus forming the anterior vagal trunk (Câmara & Griessenauer, 2015). The vagus nerve provides a link between the central nervous system and internal organs including the gastrointestinal (GI), with 80-90% of vagus fibers transmitting signals from the GI tract to the CNS (afferent fibers) and 10-20% transmitting signals in the opposite direction (efferent fibers) (Tubbs et al., 2015). Physiologically, the vagus nerve is part of parasympathetic nervous system, secretes acetylcholine from its presynaptic terminals and interact with other organs to conduct important functions like maintaining normal heart rate and digestion (Hamill & Shapiro, 2004).

Through the vagus nerve, gut microbiota help to maintain behavioral homeostasis, and supplementary administration of some specific strains of probiotics has been demonstrated to have beneficial effects on central nervous system, which do not show similar level of effects on vagotomized animals. Vagotomy is the surgical procedure which involves cutting or removing part of the vagus nerve. Subdiaphragmatic vagotomy, for it rarely affect heart and lung functions, was widely used to treat peptic ulcer disease (PUD) since introduced by Dragstedt in 1945 (Dragstedt, 1945). Among recent decades, in the field of animal research, vagotomy was found to be a practical intervention that could break the integrity of microbiota-gut-brain axis, and results showed that vagotomy had impact on animal's behavior, mood and the development of neurological disorders (Bravo et al., 2011, Sgritta et al, 2019). Our lab has used vagotomy as an intervention to

study the neural linkage between peripheral immune system and CNS. Our lab previously found that continuous oral feeding of *Lactobacillus rhamnosus* JB-1 had anxiolytic and anti-depressant effect on male BALB/c mice, however, same changes were not seen in vagotomized group (Bravo et al., 2011b). Further study of our lab found that a single dose of JB-1 could cause rapid activation of distinct brain regions by analysis of the level of c-Fos expression, and vagotomy abolished c-Fos immunoactivity in most previously responsive regions (Bharwani et al., 2020). It is already known that peripheral inflammation would affect brain and cause behavioral alterations, and that the vagus nerve is able to inhibit this inflammatory process. A dose of IL-1β or LPS that induced sickness behaviour in sham-operated animals was no longer able to decrease social exploration in vagotomised rats and mice. Vagotomy also blocked the suppressive effects of LPS on food-motivated behaviour in mice (Laye et al., 1995; Luheshi et al., 2000; Wieczorek, Swiergiel, Pournajafi-Nazarloo, & Dunn, 2005). Vagotomy could lead to increased immobility time and reduced immobility latency of tail suspension test in C57BL/6 mice, suggesting vagal pathway is important in maintenance of level of depressive behaviors (J.-E. Ghia, Park, Blennerhassett, Khan, & Collins, 2011). Another study demonstrated that vagotomized rats spent significantly less time in open arms of elevated plus maze, which is an indicator of anxiety-like behavior (Sudakov, Bashkatova, Kolpakov, & Chernyaeva, 2012). Moreover, probiotics are not the only treatments that rely on the vagus nerve integrity to exert their effects. Drugs including SSRIs (selective serotonin reuptake inhibitors) also need the vagus nerve

to alter brain function. Indeed, vagotomy could block the anti-depressant effects of sertraline and fluoxetine, members of SSRI family, compared to non-surgical mice (Neufeld et al., 2019). All these evidences suggest that vagus nerve is essential in the gut-brain axis, in the aspect of maintenance of normal behaviors. Furthermore, to fulfill the map of gut-brain axis, we have to explore whether vagus nerve has inhibiting ability to limit the microglial activation. In aged mice or APP/PS1 mice, Kaczmarcyzk et al. found that external non-invasive vagus nerve stimulation could make strong morphological changes in microglia, from a neuro-destructive to a neuro-protective phenotype, whereas microglia from young animals were morphologically unaffected (Kaczmarczyk, Tejera, Simon, & Heneka, 2018). However, it is important to determine whether the vagus nerve is essential in regulating microglial activation in non-stressed states; thus, we include a study focused on changes in microglia in vagotomized mice in this thesis.

Overall, exposure to stress leads to psychiatric disorders in both human beings and animals. Probiotic treatment, by changing the components of gut flora, could be a promising therapeutic strategy for it conducts systemically homeostatic effects through microbiota-gut-brain axis, mainly through inhibition of inflammation and activation of vagus nerve. This thesis is going to focus on the modulatory mechanisms of *L. rhamnosus* JB-1 on microbiota-gut-brain axis, especially the crosstalk between the periphery (immune, endocrine systems and vagus nerve), with central nervous system, and its impacts on behaviors.

CHAPTER 2. HYPOTHESIS AND AIMS

Central hypothesis: Probiotic treatment, by regulating microbiota-gut-brain axis, conducts behavioral modulatory effects on animals suffered from stress under specific situations. To clarify this, I **hypothesise** that 1) Early-stage administration of JB-1 or selective serotonin reuptake inhibitor directly after traumatic stress changes the social behavioral and immune function of mice; 2) $CD4^+CD25^+$ regulatory T cells are essential in *Lactobacillus rhamnosus* JB-1 induced anxiolytic and anti-depressive effects; 3) The integrity of vagus nerve is necessary in JB-1 induced maintenance of immune and neural balance through inhibition of microglial activation. These hypotheses are addressed by the following three aims.

Aims:

1. Elucidate effects of early-stage continuous administration of JB-1 and sertraline in mice suffered from chronic social defeat.
2. Explore the necessity of $CD4^+CD25^+$ regulatory T cells in JB-1 induced behavioral changes and determine the mechanism of JB-1 by adoptive transfer of $CD4^+CD25^+$ regulatory T cells.
3. Identify the necessity of the vagus nerve in central and peripheral immunoregulation in microbiota-gut-brain axis, through a study focusing on microglia changes after vagotomy and JB-1 consumption.

CHAPTER 3.

Increased persistence of avoidance behaviour and social deficits with *L.rhamnosus* JB-1 or selective serotonin reuptake inhibitor treatment following social defeat.

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Abstract

Chronic social defeat (CSD) in mice has been suggested as a model for studying post-traumatic stress disorder (PTSD). Our previous work indicated that exposure to *L. rhamnosus* JB-1 (JB-1) during CSD can attenuate subsequent behavioural and immune disruption, suggesting a potential for microbe based therapeutic approaches in PTSD.

In the current study, we assessed the ability of JB-1 to mitigate the behavioral consequences of CSD when treatment is instigated in the early post-stress period and compared the probiotic effects with those of the selective serotonin reuptake inhibitor (SSRI) sertraline. JB-1 or sertraline were administered orally 48 hours following 10-days of CSD in male C57BL/6 mice.

Contrary to our hypothesis of a beneficial effect, 30 days of treatment with either JB-1 or sertraline increased the persistence of both aggressor avoidance and reduced sociability in defeated mice. This was accompanied by lower hippocampal mRNA expression for genes related to fear memory. Defeated mice treated with either JB-1 or sertraline also exhibited systemic immune changes, with a decrease in Th1 cells, activated monocytes, and the monocyte chemoattractant CCL2. This study identifies potentially detrimental effects of both JB-1 and sertraline if administered in the early post-trauma period and suggests caution should be applied when considering psychobiotic or SSRI based approaches for early intervention in trauma related psychiatric disorders.

Key Words: Chronic social defeat; Probiotics; Selective serotonin reuptake inhibitor; Hippocampus; Immune system; Post-traumatic Stress Disorder

3.1. Introduction

Post-traumatic stress disorder (PTSD) is a debilitating condition that can develop following experience of, or exposure to, severely traumatic or life-threatening events. The disorder is characterized by clusters of symptoms including involuntary and intrusive memories of the trauma, avoidance of trauma-related stimuli and social detachment (A. Shalev, Liberzon, & Marmar, 2017). In addition, PTSD is often comorbid with anxiety and depression, and those with the disorder are up to six times more likely to commit suicide (Greenberg, Brooks, & Dunn, 2015; Kessler, Borges, & Walters, 1999; Spinhoven, Penninx, van Hemert, de Rooij, & Elzinga, 2014).

Current treatment for PTSD includes cognitive behavioral therapy (CBT) and pharmacological interventions, with selective serotonin reuptake inhibitors (SSRIs) being the first-line in pharmacotherapy (Jonas et al., 2013). However, CBT has a relatively high drop-out rate in PTSD patients, especially in the population of veterans (Garcia, Kelley, Rentz, & Lee, 2011; Monson et al., 2006), while the efficacy of SSRIs is also less than optimal, with only approximately half of patients responding to treatment and full remission achieved in 20–30% (Albucher & Liberzon, 2002; Berger et al., 2009). There is a clear need for more effective strategies to treat PTSD, or to prevent onset of the disorder following exposure to trauma.

The microbiota-gut-brain axis is a term used to describe a broad set of interactions between the gut microbiota and the central nervous system which

involve endocrine, immune, and neural signalling pathways (Forsythe, Kunze, & Bienenstock, 2016). It is now established that exposure to psychological stressors can alter the composition of the gut microbiota while, conversely, alteration of the microbiota or exposure of the gut to specific bacteria can modulate brain chemistry, stress responses and anxiety/depressive-like behaviors (Leclercq, Forsythe, & Bienenstock, 2016). Furthermore, probiotic and prebiotic treatments have been demonstrated to enhance stress resilience and mitigate PTSD relevant behaviors in animal models (Bharwani, Mian, Surette, Bienenstock, & Forsythe, 2017; Fox et al., 2017; Hassell Jr et al., 2019; Tarr et al., 2015). Such observations, together with evidence of altered microbiota profiles in subjects with psychiatric conditions including major depressive disorder (MDD) and PTSD, has prompted the exploration of microbe-based approaches to mental health (Borre, Moloney, Clarke, Dinan, & Cryan, 2014).

Oral treatment with *Lactobacillus rhamnosus* JB-1 (JB-1) has been demonstrated to lead to changes in neurotransmitters in the brains of mice and to have anxiolytic and antidepressant-like activity (Bravo et al., 2011; Janik et al., 2016). We previously demonstrated that feeding JB-1 prior to stress exposure could attenuate behavioural deficits and systemic immune alterations induced by CSD, an animal model with features of PTSD (Bharwani et al., 2017). This previous study suggested a potential prophylactic role for beneficial bacteria in mitigating the detrimental effects of subsequent stress exposure in mice. Also, in the same study, we found that 28-day administration of JB-1 did not lead to significant

differences in behaviors including sociability and aggressor-approach avoidance in mice in the absence of social defeat. However, the ability of the probiotic treatment to influence brain and behaviour when given following social defeat, and thus its potential as a treatment or early post-exposure intervention for stress and trauma related disorders, has not been assessed.

In the current study, we investigated the effects of post-defeat treatment with JB-1 on behavior, molecular alterations in the hippocampus, and immune changes in the mouse CSD model. We compared the effects of JB-1 to those of sertraline, an SSRI that is FDA approved for use in treatment of PTSD.

3.2. Methods

3.2.1. Animals. Male C57BL/6 mice (6-8 weeks old) and CD-1 (retired breeders) were acquired from Charles River (Montreal, Canada). All mice were acclimated for 1 week before the instigation of experiments, in standard conditions (12-hour light-dark cycle, 5 am - 5 pm). All mice were fed *ad libitum* with standard chow and water. The CSD experiment was conducted in 4 cohorts (3-4 mice per group per cohort), and the non-defeat experiment was conducted in 2 cohorts (4 mice per group per cohort). All mice were handled for three days before 10-day CSD or control treatment, and the intervention and behavior tests were conducted by same researcher. All experiments followed Canadian Council on Animal Care guidelines and were approved by the McMaster Animal Research Ethics Board.

3.2.2. Chronic social defeat. CD-1 aggressors were screened and chronic social defeat was conducted according to a standard protocol described by Golden et al (Golden et al., 2011). Briefly, CD-1 aggressors were housed singly in rat cages (with a clear perforated Plexiglas divider in the middle and normal bedding) 24 hours before the first session of defeat for acclimation. On the first test day, a C57BL/6 intruder was placed into the CD1 aggressor compartment to allow 5 minutes of contact with the aggressor and then moved to the opposite compartment, with olfactory, visual and auditory contacts allowed. This defeat procedure was repeated for ten days, with the intruder placed into a novel aggressor cage daily. For the C57BL/6 mice in all non-defeated groups, two C57BL/6 mice were housed in the same cage separated by a divider as described above, and the partner of each was changed daily. After the final session was completed, all intruders were housed singly. Only the susceptible mice (with an interaction ratio <1 according to the aggressor-approach avoidance screening carried out 1 day after the final session of defeat) were used for further treatment and tests.

3.2.3. JB-1 and sertraline administration. 48 hours following the final defeat session, mice in the JB-1 treated group were administered 1×10^9 CFU of *Lactobacillus rhamnosus* JB-1 (a gift from Alimentary Health Ltd., Cork, Ireland) in 200 µl of phosphate-buffered saline (PBS) by oral gavage as previously described (Bharwani et al., 2017). Non-JB-1 treated animals received an equivalent volume

of PBS via gavage, including those in the SSRI-treated groups. SSRI treated group received sertraline (6 mg/kg, dissolved in tap water, MilliporeSigma Canada Co., Oakville, Canada), via a 50 ml conical centrifuge tube with fitted sipper, while all animals not in the sertraline treatment group received tap water via the same method. We have previously demonstrated that this dose of sertraline and treatment protocol has antidepressant-like effects in mice (K.-A. M. Neufeld et al., 2019). All treatments were provided daily until the end of the experiment.

3.2.4. Behavioral tests. Behavioral tests were conducted as previously described (Bharwani et al., 2017; Bravo et al., 2011). All intruders were screened for aggressor avoidance behaviour 1 day after the final session of social defeat and then randomly assigned to their treatment groups. Following 18 days of treatment, the mice were tested in the open field test (OFT), light-dark test (LDT), elevated plus maze (EPM), 3-chamber sociability test and aggressor avoidance test. Interaction ratio was applied as result in aggressor avoidance test, calculated by time spent in aggressor area/time spent in empty area. A 4-day interval was inserted between each test in order to reduce the impact of previous tests on behavior. All behavioral tests were conducted during the dark phase under normal light conditions and with a 1h period of habituation in the testing room. OFT, LDT and EPM results were recorded by Motor Monitor software (Kinder Scientific, Poway, Canada). The results of 3-chamber sociability test and aggressor approach-avoidance test were recorded by EthoVision XT software (Noldus,

Leesburg, Canada). The apparatus was thoroughly cleaned with distilled water and dried between animals tested.

3.2.5. Flow Cytometry. Mice were euthanized 2 days following the final behavioral test using small animal guillotine. A single cell suspension of splenocytes was made after tissue harvest and lysis of red blood cells. Splenocytes ($1x10^6$) were stained for regulatory T cells (CD3-APC-eFluor780, CD4-FITC, CD25-APC, Foxp3-PE), monocytes (CD11b-PE, Ly6C-APC, CCR2-FITC, CX3CR1-PerCP/Cy5.5) and dendritic cells (CD11c-APCeFluor780, CD80-PerCP, CD86-APC, MHC II-FITC). Splenocytes stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin, were stained for markers of Th1 and Th2 cells (IFN-γ-APC, IL-4-PerCP-eFluor710). Cells were stained by extracellular antibodies at first, then cytofix was applied and followed by staining with intracellular antibodies. All conjugated antibodies were from Invitrogen™, ThermoFisher Scientific, USA, and diluted by 1:200 before adding to the plates. The flowcytometry results were analysed by FlowJo software (Flowjo LLC, USA), with doublets and cell debris excluded by FSC and SSC gating.

3.2.6. Cytokine/chemokine analysis. Serum was collected from trunk blood directly after euthanization from common carotid artery. The levels of interleukin (IL) -1a, -1b, -2, -4, -5, -6, -10, -12, -13, -17A, chemokine (C-X-C motif) ligand-1, -2, -5, C-C motif ligand-2 (CCL-2), tumor necrosis factor-alpha (TNF-α), and

interferon-gamma (IFN-γ) were determined through Multiplex Cytokine/Chemokine Analysis (Eve Technologies, Calgary, Canada). Data is presented as fold changes in the figures and tables.

3.2.7. RT-qPCR. Hippocampus as a whole was collected and RNA was extracted using *mir*Vana™ miRNA isolation kit (Thermofisher Scientific, USA) at the day of euthanization by dissection. The quality of the extracted RNA was analysed using NanoDrop® Spectrophotometer ND-1000 (Thermofisher Scientific, USA) and DNA contaminants removed using Invitrogen TURBO DNA-*free*™ kit. cDNA was created using the Applied Biosystems High Capacity cDNA Reverse Transcription kit (Thermofisher Scientific, USA).

Primers were chosen based on previous studies (Bravo et al., 2011; Leclercq et al., 2017; K. Neufeld, Kang, Bienenstock, & Foster, 2011) and all primer sequences are listed in Supplementary Table S1. PowerUP™ SYBR®Green Master Mix (Applied Biosystems, Life Technologies, USA) containing ROXTM Passive Reference Dye was mixed with cDNA and the appropriate primers. The qPCR reaction was carried out in fast mode (uracil-DNA polymerase activation 50°C, 2 min; Dual-Lock™ DNA polymerase 95°C, 2s; denaturation 95°C, 1s; annealing/extension 60°C, 30s; number of cycles: 40) using QuanStudio3TM (Applied Biosystems). The transcripts were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and quantified using the

ΔΔCt method, with related fold change expressed as $2^{(-\Delta\Delta Ct)}$. Each sample was run as a triplicate.

3.2.8. Statistical analysis. Data were analysed using the software GraphPad Prism 8.0 by t test (for comparisons between non-defeat control and defeat control groups) and one-way ANOVA with Bonferroni-corrected post hoc tests (for comparison between treatment groups and appropriate vehicle control). In figures results are illustrated as mean ± standard error of the mean (SEM) unless otherwise stated. *p* value smaller than 0.05 was considered as statistically significant. Effect size was analysed as partial eta squared (η^2p) for main effects, and Hedge's G (g) was applied for post-hoc analysis.

3.3. Results

3.3.1. JB-1 and sertraline treatment both increase persistence of aggressor avoidance and reduced sociability in mice when administered following chronic social defeat.

The timeline of the experiments is illustrated in Fig. 1.

Animals were initially divided into 2 groups of non-defeated (n=12) and defeated (n=36) mice. Following CSD, an aggressor avoidance test was used to confirm that mice were susceptible to social defeat prior to inclusion in post-defeat treatment groups, with an interaction ratio of <1 considered as avoidance behaviour (Golden, Covington III, Berton, & Russo, 2011). 48 hours following the

final defeat session, defeated mice displayed avoidance behavior which was significantly different from non-defeat control (non-defeat control: 2.77±0.40, max: 6.65, minimum: 1.34, defeated: 0.10±0.02, max: 0.45, minimum: 0.00, g=3.40, $p<0.0001$) (Fig. 2A). Having confirmed avoidance behaviour in all defeated mice, these animals were then randomly assigned to the 3 different treatment groups (control, JB-1 or sertraline treated).

Following 18 days of treatment (day 21 post-defeat), mice underwent behavior tests. In the OFT the defeated control group spent significantly less time in the center zone compared to the non-defeated group (non-defeat control: 496.08±64.92 s, defeat control: 278.81±39.00 s, g=1.08, p=0.0111). Treatment with JB-1 had no significant effect on the behaviour of defeated mice in the OFT, however sertraline increased the time defeated mice spent in the center zone as compared to defeat control mice (defeat sertraline: 566.87±56.96s, $F(2, 33)=4.294$, $\eta^2p=0.21$, g=1.70, p=0.0001), indicative of an anxiolytic effect of the SSRI (Fig. 2B). On post-defeat days 24 and 28 we conducted the LDT and EPM respectively. There was no significant difference between the behavior of defeated and non-defeated mice in either test and no effect of treatment with either JB-1 or sertraline (Fig. 2C& 2D).

After 30 days of treatment (post-defeat day 32), all mice were tested in the 3-chamber sociability test and retested for aggressor-approach avoidance behaviour. The sociability test demonstrated a significant increase in social behaviour in defeated mice as compared to non-defeat control (non-defeat control:

274.76±17.33s, defeat control: 378.01±26.43s; g=1.38, p=0.0028) at post-defeat day 32. This finding is in contrast to a cohort of mice tested for social behavior 1 day after social defeat, where a significant impairment in social interaction was observed (See Supplementary Fig. S1). Compared to the defeat control group at 32 days post-defeat, mice treated with either JB-1 or sertraline spent significantly less time in the mouse chamber ($F(2, 32)=1.765$, $\eta^2p=0.10$, JB-1: 146.94±44.83s, g=1.81, p=0.0006; sertraline: 175.42±40.10s, g=1.73, p=0.0025) (Fig. 2E). In the second aggressor approach avoidance test there was no longer a significant difference in the mean interaction ratio between the defeat and non-defeat controls (non-defeat control: 1.34±0.25, max: 3.07, min: 0.37; defeat control: 1.07±0.32, max: 2.86, min: 0.00; g=0.27, p=0.5216), however the difference was maintained in both the JB-1 and sertraline treated mice, with both these groups also demonstrating significantly lower interaction ratios than defeat control mice. ($F(2, 32)=21.44$, $\eta^2p=0.57$, JB-1: 0.047±0.016, g=1.40, p=0.0010; sertraline: 0.21±0.070, g=1.12, p=0.0058) (Fig. 3A). Comparison of the time spent in interaction zone between tests before and post-treatment by paired t test showed a significant decrease in non-defeat control group, but a significant increase in defeat control group, which was not shown in defeat JB-1 or sertraline group (non-defeat control: g=1.19, p=0.0151; defeat control: g=1.28, p=0.0145; defeat JB-1: g=0.36, p=0.04280; defeat sertraline: g=0.55, p=1535) (Fig. 3B). Moreover, 32 days after defeat, 5 of 11 (45.4%) of mice in defeat control group showed an interaction ratio > 1, which was not observed in mice treated with either JB-1 or sertraline (See

Supplementary Fig. S2A). The locomotor ability was not changed among groups according to basic movements of OFT (Supplementary Fig. S2B).

To determine if the effects observed in JB-1 and sertraline groups occurred independently of CSD we examined additional cohorts of mice, housed identically to the CSD mice but without exposure to aggressors. In these non-defeated mice, we did not find any significant difference in behaviour following treatment with JB-1 or sertraline. Fig. 2F to 2I, and Fig. 3C. As with our initial non-defeated cohorts, the interaction ratio of aggressor avoidance was reduced between the first and second test (control: before treatment: 3.583±0.674, post-treatment: 1.406±0.215, g=1.54, p=0.0131; JB-1: before treatment: 3.119±0.512, post-treatment: 1.252±0.150, g=1.75, p=0.0031), while a trend of reduction in sertraline group (before treatment: 2.883±0.767, post-treatment: 1.206±0.162, g=1.07, p=0.0718) (Fig. 3D). Also, the locomotor ability was not affected by either JB-1 or sertraline (Supplementary Fig. S2C).

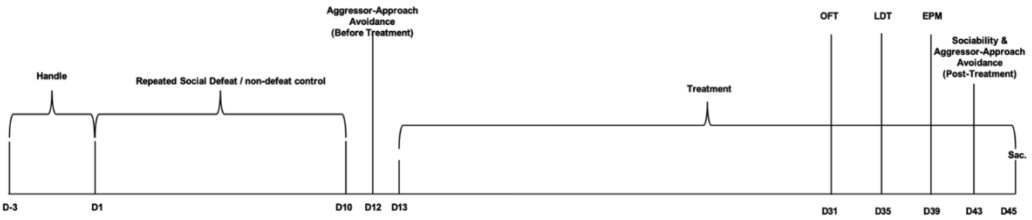

Figure 1. Timeline of the experimental protocol (OFT: open field test; LDT: light-dark test; EPM: elevated plus maze).

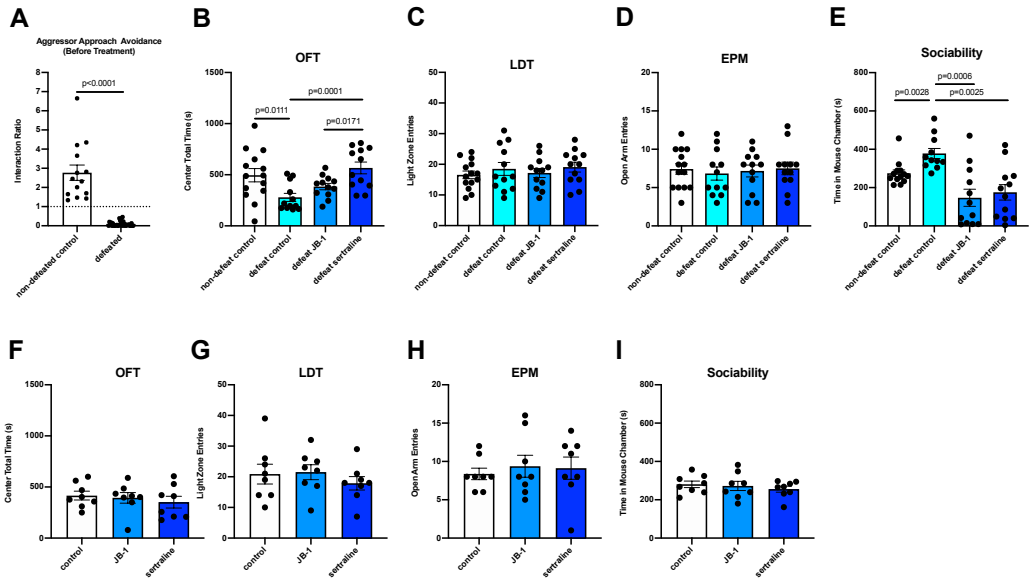

Figure 2. Anxiety-like and social behavior in CSD (A-E) and non-defeated mice (F-I) following treatment with JB-1 or sertraline. (A) Interaction ratio (time spent in aggressor area/time spent in empty area) of aggressor approach avoidance test, before treatment (n=14 in non-defeat control group, n=36 in defeated group). **(B)** Time spent in center area of open field test. (n=14 in non-

defeat control group, n=12 in each of other groups). **(C)** Entries into light zone of light-dark test (n=14 in non-defeat control group, n=12 in each of other groups). **(D)** Entries into open arm of elevated plus maze (n=14 in non-defeat control group, n=12 in each of other groups). **(E)** Time spent in mouse chamber of 3-chamber sociability test (n=13 in non-defeat control group, n=12 in each of other groups). **(F)** Time spent in center area of open field test (n=8). **(G)** Entries into light zone of light-dark test (n=8). **(H)** Entries into open arm of elevated plus maze (n=8). **(I)** Time spent in mouse chamber of 3-chamber sociability test (n=8).

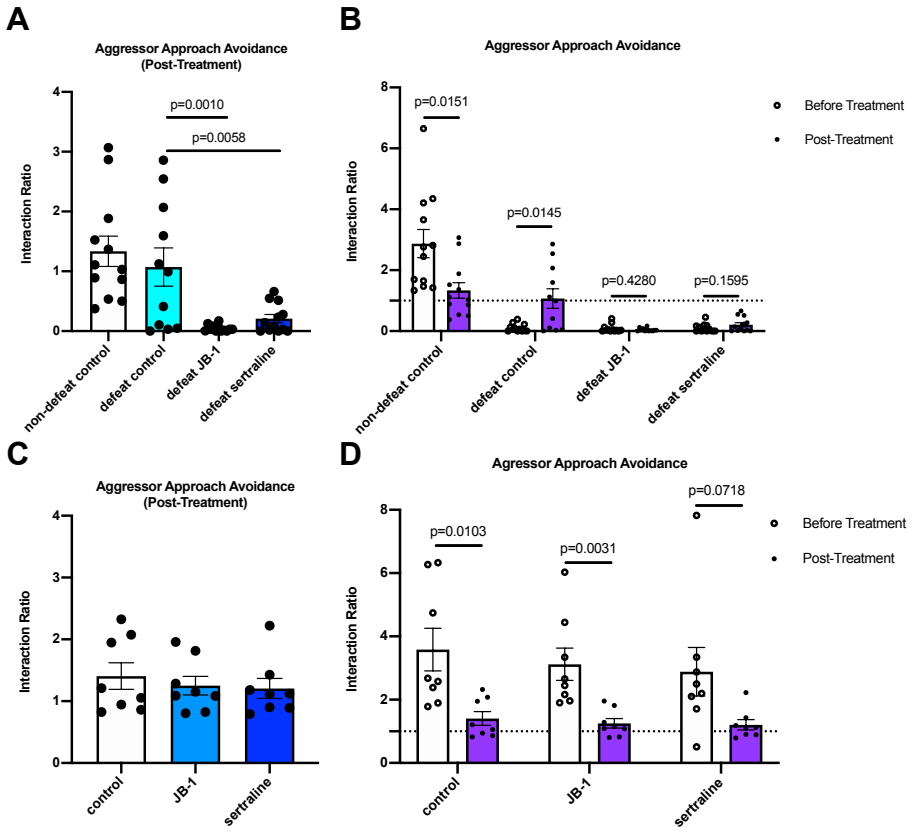

Figure 3. Post-treatment interaction ratio in the aggressor avoidance test and comparison of interaction ratio, pre and post-treatment by paired t test, in CSD **(A, B)** exposed mice (n=11-12), and non-defeated **(C, D)** cohorts (n=8).

3.3.2. JB-1 and sertraline alter hippocampal gene expression in mice exposed to social defeat.

We assessed gene expression in the hippocampus of mice 32 days following CSD, with and without post-defeat JB-1 and sertraline treatment; targeting genes related to stress response, social behavior and memory that had been previously identified as being modulated by JB-1 in other models (Bravo et al., 2011; Kayyal et al., 2020; Leclercq et al., 2017). For corticotropin-releasing hormone receptor subtypes (CRHR-1 and -2) and arginine vasopressin (AVPR-1a and -1b) in the hippocampus, we found that mice treated with JB-1 or sertraline had significantly lower expression of CRHR-1 mRNA relative to mice in defeat control group (F (2, 15)=1.391, η^2p=0.16, defeat control: 0.81±0.097, JB-1: 0.37±0.049, g=2.32, p=0.0020; sertraline: 0.44±0.061, g=1.88, p=0.0069) (Fig. 4A). There was no significant difference in CRHR-2 or AVPR-1a and -1b gene expression (Fig. 4B-D).

Defeated mice treated with JB-1 and sertraline also had reduced expression of both mineralocorticoid and glucocorticoid receptors (MR and GR) compared to defeat control (MR: F (2, 15)=0.098, η^2p=0.01, defeat control: 1.16±0.084, JB-1:

0.70±0.11, g=1.93, p=0.0148; sertraline: 0.73±0.10, g=1.84, p=0.0227) (Fig. 4e) (GR: F (2, 14)=1.198, η^2p=0.15, defeat control: 1.20±0.080, JB-1: 0.73±0.051, g=2.96, p=0.0051; sertraline: 0.70±0.10, g=2.23, p=0.0019) (Fig. 4F).

There was no significant change in relative BDNF expression in control defeated compared to non-defeat mice 32 days after defeat. However, defeated mice treated with JB-1 had significantly lower BDNF expression (F (2, 15)=1.403, η^2p=0.16, defeat control: 0.84±0.13, JB-1: 0.43±0.053, g=1.69, p=0.0173) (Fig. 4G). For gamma-aminobutyric acid receptor subunit A2 ($GABA_{A2}$) and subunit B1b ($GABA_{B1b}$), we observed significantly lower $GABA_{A2}$ expression in JB-1 treated animals compared to defeat controls, (F (2, 14)=0.407, η^2p=0.05, defeat control: 1.05±0.12, JB-1: 0.59±0.086, g=1.80, p=0.0362) (Fig. 4H) while no differences were observed between groups in the expression of $GABA_{B1b}$ (Fig. 4I).

Treatment of non-defeated mice with JB-1 or sertraline did not result in any significant changes in expression of the genes assessed (see Supplementary Fig. S3).

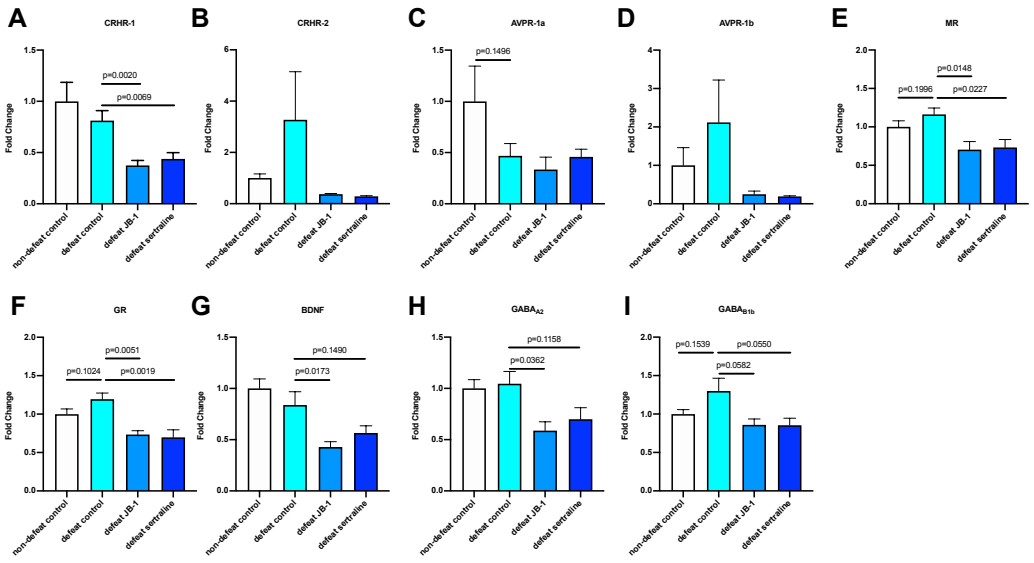

Figure 4. Stress-related gene expression in the hippocampus, represented as fold change compared to mean value of the non-defeat control group (n=6). **(A)** Corticotropin-releasing hormone receptor-1 (CRHR-1). **(B)** Corticotropin-releasing hormone receptor-2 (CRHR-2). **(C)** Arginine vasopressin receptor-1a (AVPR-1a). **(D)** Arginine vasopressin receptor-1b (AVPR-1b). **(E)** Mineralocorticoid receptor (MR). **(F)** Glucocorticoid receptor (GR). **(G)** Brain-derived neurotrophic factor (BDNF). **(H)** Gamma-aminobutyric acid receptor subunit alpha-2 ($GABA_{A2}$). **(I)** Gamma-aminobutyric acid receptor subunit beta-1b ($GABA_{B1b}$).

3.3.3. JB-1 and sertraline treatment reduced Ly6C^{hi} monocytes and dendritic cell populations, following chronic social defeat.

Activated monocytes have been recognized as a key inflammatory signal following exposure to social defeat, and increases in Ly6C^{hi} monocytes have been

demonstrated to drive anxiety-like behavior following stress exposure (Weber, Godbout, & Sheridan, 2017). In our study, we found that 32 days after CSD, the population of $CD11b^{+}Ly6C^{hi}$ cells was not significantly different between non-defeat and defeat control groups (non-defeat control: 8.39±0.55%, defeat control: 7.52±0.33%, g=0.79, p=0.2025). However, both sertraline and JB-1 treatment groups had a significantly reduced population of $Ly6C^{hi}$ monocytes (F (2, 15)=3.696, η^2p=0.33, JB-1: 5.73±0.62%, g=1.46, p=0.0238; sertraline: 5.39±0.13%, g=3.49, p=0.0072) (Fig. 5A). An increase in T regulatory cells (Treg), has been associated with the anxiolytic and antidepressant-like effects of JB-1 (Liu, Mian, Neufeld, & Forsythe, 2020). Treg were assessed based on expression of Foxp3 in $CD4^{+}$ T cells. The sertraline treatment group had a significantly smaller population of $Foxp3^{+}$ Treg when compared to defeated control (F (2, 15)=1.108, η^2p=0.13, defeat control: 8.58±0.34%, sertraline: 6.78±0.39%, g=1.78, p=0.0039) (Fig. 5B). There was no significant difference in activated dendritic cells (CD80/CD86) among groups (Supplementary Fig. S4A & S4B).

As a measure of general immune balance, stimulated splenocytes were used to assess Th1 and Th2 cell populations based on IFN-γ and IL-4 expression respectively. Defeat alone led to a significant decrease in IFN-γ expressing $CD4^{+}$ (Th1) cells with no significant effect of treatments on this decrease (t test: non-defeat control: 2.52±0.42%, defeat control: 0.96±0.42%, g=1.52, p=0.0251; one-way ANOVA: F (2, 15)=5.140, η^2p=0.41, JB-1: 0.19±0.072%, g=1.06, p=0.1207; sertraline: 0.043±0.029%, g=1.28, p=0.0529) (Fig. 5C). There was no significant

difference in the Th2 ($CD4^+IL\text{-}4^+$) cell among groups (Fig. 5D). However, the Th1/Th2 ratio, as an indicator of immune balance, showed a significant decrease after defeat (non-defeat control: 0.17±0.041%, defeat control: 0.058±0.024%, g=1.39, p=0.0367), and sertraline, but not JB-1, treatment was associated with a significant reduction compared to defeat control ($F(2, 15)=5.367$, $\eta^2p=0.42$, JB-1: 0.0103±0.0039, g=1.13, p=0.0897; sertraline: 0.0031±0.0021%, g=1.32, p=0.0438) (Fig. 5E).

There was no effect of either JB-1 or sertraline treatment on $Ly6C^{hi}$ monocytes in non-defeated mice (Fig. 5F). However, JB-1 increased, and sertraline decreased, the population of $Foxp3^+$ T cells compared the control group ($F(2, 15)=2.404$, $\eta^2p=0.24$, control: 16.08±0.68, JB-1: 18.55±0.25, g=1.96, p=0.0422; sertraline: 13.25±0.81, g=1.54, p=0.0182) (Fig. 5G). JB-1 or sertraline treatment alone had no effect on Th1 and Th2 cells (Fig. 5H-5J) or $CD80^+$ and $CD86^+$ dendritic cells (Supplementary Fig. S4C & S4D).

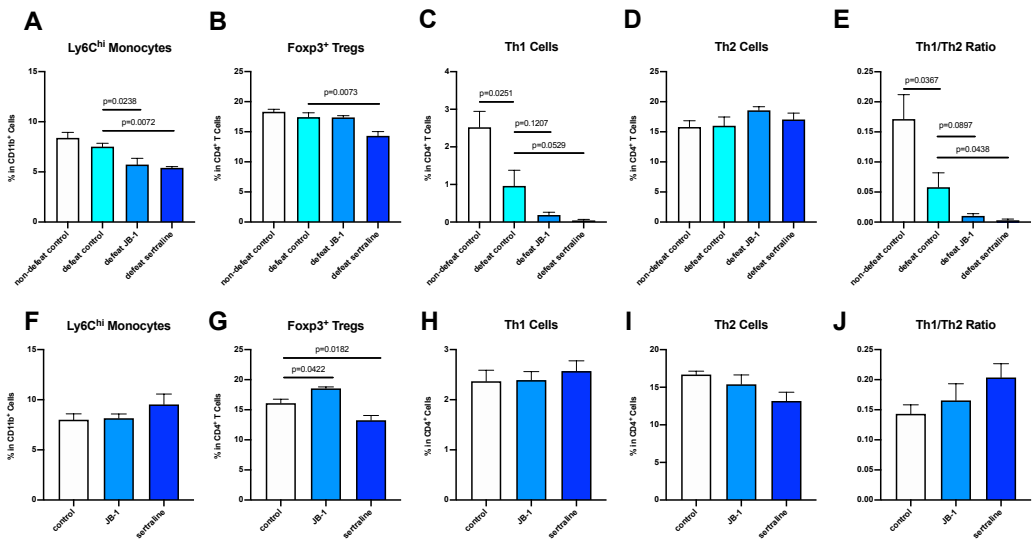

Figure 5. **Flowcytometry analysis of splenocytes from the CSD exposed (A-E) and non-defeated (F-J) mice following treatment with JB-1 or sertraline (n=8),** showing Ly6C^{hi} monocytes **(A, F)**, Foxp3^{+} Tregs. **(B, G)**, Th1 cells (IFN-γ^{+}IL-4^{-}). **(C, H)**, Th2 cells (IFN-γ^{-}IL-4^{+}) **(D, I)**, and Th1/Th2 ratio (percentage of Th1/percentage of Th2) **(E, J)**.

3.3.4. Serum CCL-2 was decreased after JB-1/ sertraline treatment on mice after chronic social defeat.

In assessing serum levels of 18 cytokines/chemokines in mice following CSD, we found that relative levels of CCL-2, a major chemoattractant for activated monocytes, were significantly decreased in both JB-1 and sertraline treatment groups compared to defeat control. ($F(2, 29)=4.390$, $\eta^2p=0.23$, defeat control: 0.649±0.145, JB-1: 0.284±0.034, $g=1.02$, $p=0.0260$; sertraline: 0.222±0.036,

g=1.22, p=0.0064) (Fig. 6A). In addition, sertraline treatment led to a significant reduction in IL-6 compared to defeated control (F (2, 28)=5.237, η^2p=0.27, defeat control: 2.552±0.775, sertraline: 0.523±0.147, g=1.18, p=0.0119) (Fig. 6B), The results of additional cytokines/chemokines analysed are shown in Supplementary Table S2.

In the non-defeated cohorts, we did not find significant difference among groups in CCL-2 (Fig. 6C). However, we did find JB-1 treatment alone led to significant reduction of IL-6 (F (2, 20)=2.629, η^2p=0.21, control: 1.000±0.2440, JB-1: 0.4295±0.0854, g=1.21, p=0.0434) (Fig. 6D). No significant difference was found in the other cytokines and chemokines assessed (see Supplementary Table S3).

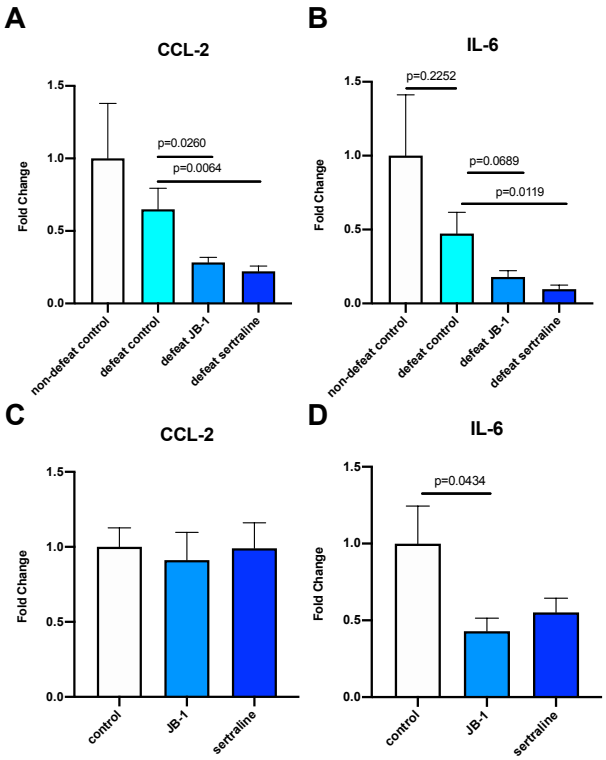

Figure 6. Serum Chemokine (C-C motif) ligand-2 (CCL-2) an Interleukin-6 (IL-6) levels following sertraline or JB-1 treatment in (A, B) CSD exposed (n=10-12) and (C, D) non-defeated (n=8) mice. Data is expressed as fold change compared to corresponding non-defeated control.

3.4. Discussion

Here we report that treatment with JB-1 or sertraline, initiated 48 hours following chronic social defeat, led to a greater persistence of aggressor avoidance and deficits in social behavior compared to control defeated mice. Treatment with JB-1, post-defeat, had no effect on anxiety-like behaviour. The findings contrast those of our previous study which demonstrated that JB-1 treatment concurrent with CSD results in attenuation of both social behavior deficits and anxiety-like behaviors (Bharwani et al., 2017). Thus, it appears that, depending on the temporal relationship between stress exposure and treatment with the bacteria, microbes identified as having anxiolytic/anti-depressant like effects have the potential to potentiate detrimental effects of traumatic events and/or stress.

For mice in the defeat control group, the reduction in social interaction that occurs immediately following stress exposure was not observed 4 weeks later. In the same 4-week period, aggressor avoidance behavior was lost in approximately 50% of these mice, suggesting some mice do not develop long-term fear memory in this CSD model (Bharwani et al., 2017). In a previous study we identified that anxiety associated behaviours which occur immediately following CSD are not

apparent 3 weeks later but did not observe a loss in aggressor avoidance behaviour (Bharwani et al., 2017). This difference may be due to the additional 10 days of recovery time in the current protocol. However, treatment with either JB-1 or sertraline resulted in persistence of attenuated social interactions and avoidance behaviour in all the mice tested. That this is a persistence of the effects of CSD rather than actions of the treatment alone is supported by the observation that neither JB-1 nor sertraline altered these behaviours in non-defeated mice. We also noted that in non-defeated mice, regardless of treatment, there was a reduced interaction ratio between the first and second aggressor avoidance test. This likely reflects that in non-defeated mice the initial exposure is one of social novelty, which is lessened in the second exposure.

Using a similar CSD model, Ishikawa et al. found a comparable decrease in percentage time spent in aggressor avoidance zone between 1 day and 4 weeks following stress exposure (Ishikawa, Uchida, Kitaoka, Furuyashiki, & Kida, 2019). However, Hammamieh et al. demonstrated that fear memory persisted 4 weeks after repeated exposure to conspecific trained aggressors (Hammamieh et al., 2012). The difference between persistence of behaviours may be due to the distinct models used (chronic social defeat, 5min daily for 10 days by retired-breeder CD1 mice, vs cage-within-cage intruder exposure, 6h daily for 5-10 days by 6-week old SJH albino mice).

There have been few previous studies addressing the use of microbe-based treatments following stress exposure. Hassell et al. reported that rats immunized

with heat killed *M. vaccae*, one day following fear conditioning, did not show altered fear expression but did have improved fear extinction (Hassell Jr et al., 2019). It is difficult to compare the work of Hassell et al. with our current study as we used a different model (fear conditioning vs CSD), different species (rat vs mouse) and studied natural loss of the fear response rather than active extinction. Furthermore, it is possible there are distinct mechanisms of action and effects on brain when comparing subcutaneous administration of *M. vaccae* with oral treatment of JB-1.

With regard to sertraline effects, our observations may be in keeping with previous studies indicating that SSRI treatment can modulate fear conditioning (Burghardt, Sigurdsson, Gorman, McEwen, & LeDoux, 2013; Montezinho et al., 2010). Montezinho et al. demonstrated that escitalopram differentially affected distinct stages of contextual fear conditioning. Escitalopram significantly decreased the conditioned responses when administered 30 mins before the recall test. However, when applied immediately after acquisition, during consolidation, it enhanced freezing time during fear recall, indicating that escitalopram potentiates memory consolidation (Montezinho et al., 2010). Moreover, serotonin transporter knockout has been correlated with retention of contextual fear memory (Hartley et al., 2012). Such effects are thought to be due to an important role for the serotonergic system in learning and memory processes, particularly during the encoding and consolidation phases (Meneses & Liy-Salmeron, 2012). The time window immediately following fear conditioning or trauma exposure seems to be critical to the fear enhancing effects of SSRIs as Wang et al. demonstrated that

administration of paroxetine after a space of one week following conditioned fear stress (electric shock) combined with single-prolonged stress (immobilization) reduced anxiety-like behaviour and fear conditioned freezing in mice (H. Wang et al., 2012). There is evidence that environmental context is critical to the action of SSRIs. Alboni et al. demonstrated that previously stressed mice treated with fluoxetine in an enriched environment improved their depression-like phenotype whereas those treated in a stressful condition demonstrated more marked depressive behaviours than controls (Alboni et al., 2017). Such findings support the "undirected susceptibility to change' hypothesis, which posits that SSRI treatment does not drive changes in mood per se but increases brain plasticity allowing for change driven by the quality of the environment (Alboni et al., 2017; Branchi, 2011). Such increased plasticity in a period soon after defeat may help in the persistence of aggressor related memories and suggests future studies should examine neurogenesis in response to sertraline and JB-1 following CSD.

The effects of JB-1 and sertraline following CSD may also reflect the clinical picture in relation to early-stage pharmacological prophylaxis for PTSD. Such approaches have had, at best, mixed results. Early findings by Pitman et al. on propranolol (a beta-adrenergic blocker) found that patients treated with propranolol had reduced negative responses during script-driven imagery of the traumatic event than placebo, although overall there was no significant difference in the number of subjects that went on to develop PTSD (Pitman et al., 2002). However, Shalev et al. found that early intervention in trauma exposed subjects with the SSRI

escitalopram, led to a slightly higher rate in PTSD prevalence (A. Y. Shalev et al., 2012) while a more recent clinical trial found that early administration of escitalopram had no effect on PTSD development in individuals exposed to trauma (Zohar et al., 2018).

In the current study, we limited our investigation of the brain to gene expression in the hippocampus. Hippocampal changes after exposure to traumatic events have been correlated with chronic psychiatric symptoms (Bonne et al., 2001; Schuff et al., 1997; Shin et al., 2004). The expression of the CRHR-1 and -2, and AVPR-1a and -1b in the hippocampus have been demonstrated to be related to HPA axis activation and stress induced depressive and anxiety-like behavior (Neumann & Landgraf, 2012; Schmidt et al., 2018). Moreover, changes in CRHR-1 expression have been linked with fear conditioned memory and cognitive deficits (Thoeringer et al., 2012; X.-D. Wang et al., 2011). Thus, our observation that defeated mice treated with either JB-1 or sertraline had reduced CRHR-1 in the hippocampus may be functionally related to the behavioral difference in aggressor-approach avoidance test, as an indicator of fear conditioned memory.

Exposure to CSD followed by treatment with either JB-1 or sertraline, resulted in reduced expression of hippocampal MR and GR. Both receptor types have been implicated in the consolidation of fear memory and impaired fear extinction. MR and GR in hippocampus regulate the hypothalamic pituitary adrenal

axis (HPA axis) and are thus involved in the modulation of stress response, including fear, memory and anxiety (de Kloet, Oitzl, & Joëls, 1993; Gesing et al., 2001; Liberzon, Lopez, Flagel, Vazquez, & Young, 1999). Lower MR functionality can result in an increased susceptibility for negative stress responses (ter Heegde, De Rijk, & Vinkers, 2015). Brinks et al. found that forebrain MR gene inactivated mice had higher arousal and less locomotor activity, as well as more freezing in cue-related fear behavior test (Brinks, Berger, Gass, De Kloet, & Oitzl, 2009). In relation to GR, the ameliorating effects of cannabinoid receptor agonist on contextual fear extinction are prevented by blocking GR in the hippocampus, indicating that GR activation is positively correlated with fear extinction (Ganon-Elazar & Akirav, 2013).

Changes in hippocampal BDNF expression may also play an important role in mediating the observed effects of JB-1 on behaviour. Our data suggest that post-CSD administration of JB-1 leads to reduced BDNF expression in the hippocampus. BDNF has been closely linked to stress responses and memory of fear experience, with stronger fear memory correlated with lower levels of BDNF in hippocampus (Smith, Makino, Kvetnansky, & Post, 1995) while intrahippocampal administration of BDNF induces extinction of conditioned fear even in the absence of extinction training (Peters, Dieppa-Perea, Melendez, & Quirk, 2010). It is possible that reduced levels of BDNF in the hippocampus in turn inhibit or retard loss of fear related memories, maintaining aggressor avoidance and social behavior deficits.

Overall, our results indicate that both JB-1 and sertraline lead to alterations in the expression of several molecular factors involved in fear related memory and stress responses in the hippocampus of defeated mice. Such observations warrant a more detailed investigation of a potential causal relationship between the observed gene expression changes and the prolongation of CSD induced alterations in behavior.

It is established that psychological stress can lead to immunological alterations and vice versa (Hughes, Connor, & Harkin, 2016; Réus et al., 2015). While an increase in markers of inflammation has been associated with both CSD in mice (Bharwani et al., 2017) and PTSD in humans (Gill, Saligan, Woods, & Page, 2009), the causal relation between the immune system and associated behavioral changes is not well understood. The observed effects of JB-1 and sertraline on the immune system of defeated mice could generally be described as anti-inflammatory, with a decrease in Th1 cells and activated monocytes. A possible explanation of these changes could be a compensatory activation of the HPA axis following the reduction of GR/MR expression. Harris et al. found that low level of GR and MR could not only lead to retention of fear memory, but also impaired negative feedback of HPA axis, which was correlated with higher level of corticosteroid in plasma (Harris, Holmes, De Kloet, Chapman, & Seckl, 2013). HPA axis hyperactivation is linked with to a series of immunosuppressive effects including lower IFN-γ and IL-6 levels (Kovalovsky, Refojo, Holsboer, & Arzt, 2000; Viveros-Paredes, Puebla-Pérez, Gutiérrez-Coronado, Sandoval-Ramírez, &

Villaseñor-García, 2006). Further studies on hyperactivity of HPA axis could be conducted to explore the possible mechanism of compensatory hyperactivity of HPA axis after GR/MR reduction.

Monocytes are one of the major cellular components of an inflammatory response. In our study, we found that defeated mice treated with JB-1 or sertraline had fewer $Ly6C^{hi}$ monocytes, with associated lower serum levels of CCL-2, one of the major chemoattractants for monocytes. A potential causal relationship between a decrease in $Ly6C^{hi}$ monocytes and persistence of aggressor avoidance and reduction in social preference is unclear. However, it has been demonstrated that circulating $Ly6C^{hi}$ monocytes can migrate to the brain (Wohleb et al., 2014) and once in the brain these cells can influence hippocampal neurogenesis and cognition (Möhle et al., 2016). Another possibility is that the change in monocytes activation is instead related to the anxiolytic effects of sertraline and JB-1. Anxiety-like behavior induced by chronic social stress was demonstrated to be linked with higher numbers circulating activated monocytes ($CD11b^{+}Ly6C^{hi}$ cells) and greater macrophage trafficking to the brain (Weber et al., 2017). Furthermore, an increase in circulating activated monocytes has been associated with stress resilience in mice (Ambree, Ruland, Scheu, Arolt, & Alferink, 2018). While no anxiolytic effects of JB-1 were observed in the current study, possibly because of the limited anxiety like behaviour exhibited by defeated mice at the time-points studied, previous studies have indicated the ability of JB-1 to reduce both trait (Bravo et al., 2011) and state (Bharwani et al., 2017) anxiety-like behavior in mice. The relationship

between inflammation and cognition is clearly complex and investigation of the potential effects of immunomodulation on event related fear memory is warranted.

In conclusion, this study demonstrates, for the first time, that an organism previously identified as having both anxiolytic and antidepressant activities can, under certain circumstances perpetuate, rather than prevent, behavioral deficits associated with stress exposure. In addition, we demonstrate that treatment with the SSRI, sertraline, in the immediate aftermath of a chronic stressor, likely corresponding to the memory consolidation phase, can also exacerbate negative sequelae. Under both treatment conditions, such effects are associated with changes in hippocampal expression of genes associated with fear memory and modulation of the immune system, most markedly a decreased in circulating inflammatory monocytes. This data invites further exploration of the relationship between bacteria in gut and trauma or stressor related memory, particularly during the consolidation phase. The similarity of effects between JB-1 and sertraline also encourages studies focusing on possible shared mechanistic pathways between the probiotic and SSRI. In this regard, there is evidence that both JB-1 and oral SSRIs activate the vagus nerve (Bharwani et al., 2020; Bravo et al., 2011; K.-A. M. Neufeld et al., 2019; Ondicova, Tillinger, Pecenak, & Mravec, 2019; Perez-Burgos et al., 2013), and subdiaphragmatic vagotomy prevents certain effects of both treatments on the brain and behaviour of mice (Bharwani et al., 2020; Bravo et al., 2011; K.-A. M. Neufeld et al., 2019).

Given that this study identifies the potential for detrimental effects of both JB-1 and sertraline in stress/trauma related conditions, future work examining the potential ramifications of early intervention following trauma on patients at risk for development of PTSD are necessary.

Conflict of interests

The Authors declare no competing interests.

Contributors

Y.L. and P.F., designed the experiments. Y.L. and K.S. performed experiments. Y.L., K.S., A.B., M.F.M. and K.-A.M.N. analysed the data and constructed the figures. Y.L. and P.F. wrote and edited the manuscript, which was reviewed before submission by all authors.

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3.5. Supplementary Information

Target mRNA	Forward (F) and Reverse (R) Primers
GAPDH	F: 5'-TGG CCT CCA AGG AGT AAG AAA C-3' R: 5'-GGG ATA GGG CCT CTC TTG-3'
CRHR1	F: 5'-TGG TGG CCT TTG TCC TCT TC-3' R: 5'-AAA GCC GAG ATG AGG TCC C-3'
CRHR2	F: 5'-TGA CCA GCC CTT TAC CAA GGT-3' R: 5'-CCG ACT GAA AGC CAG CAT TC-3'
AVPR1a	F: 5'-GGG ATA CCA ATT TCG TTT GG-3' R: 5'-AAG CCA GTA ACG CCG TGA T-3'
AVPR1b	F: 5'-TCT ACT CTC CGT CTT AGC CTT AAC CT-3' R: 5'-CTC CAT CCA CCT GCT CCA A-3'
MR	F: 5'-ATG GAA ACC ACA CGG TGA CCT-3' R: 5'-AGC CTC ATC TCC ACA CAC AAG-3'
GR	F: 5'-AAA GGT GGC GCT TAT GTA CTT AGA G-3' R: 5'-CGT GCG GAG GCT GCA T-3'
BDNF	F: 5' CTG ACA CTT TTG AGC ACG TCA TC-3' R: 5'-CAC CCG GGA AGT GTA CAA GTC-3'
$GABA_{A\alpha2}$	F: 5'-CCA AAG ATC CTG TCC TCT CTA CCA-3' R: 5'-GGC TTC AGC TGG CTT GTT CT-3'
$GABA_{B1b}$	F: 5'-CGA GGT GAA TGG CAG TCT GA-3' R: 5'-CAC GGT TTT CCT TCT CCA ACA-3'

Supplementary Table S1. Primer sequences of brain PCR.

	non-defeat control	defeat control (p value*)	defeat JB-1 (p value**)	defeat sertraline (p value**)
IL-1a	423.4±72.5	275.4±64.3 (p=0.1422)	286.1±35.9 (p>0.9999)	277.3±39.3 (p>0.9999)
IL-1b	3.1±1.1	1.2±0.4 (p=0.0961)	1.0±0.3 (p>0.9999)	1.2±0.3 (p>0.9999)
IL-2	6.6±1.9	7.4±1.5 (p=0.7228)	8.7±1.2 (p>0.9999)	11.8±1.7 (p=0.1411)
IL-4	0.6±0.2	0.2±0.1 (p=0.0659)	0.1±0.0 (p>0.9999)	0.1±0.0 (p=0.9346)
IL-5	9.9±2.2	3.5±0.5 (p=0.0115)	6.4±1.3 (p=0.0523)	3.4±0.4 (p>0.9999)
IL-6	6.0±2.5	2.8±0.9 (p=0.2252)	1.1±0.3 (p=0.0689)	0.6±0.2 (p=0.0119)
IL-10	9.4±4.0	1.3±0.3 (p=0.0431)	2.7±0.7 (p=0.1042)	1.7±0.3 (p>0.9999)
IL-12	17.8±8.2	15.6±3.7 (p=0.8035)	11.4±2.4 (p=0.7671)	4.9±1.0 (p=0.0155)
IL-13	11.8±1.6	9.8±0.7 (p=0.2881)	7.3±0.8 (p=0.0403)	7.4±0.5 (p=0.0304)
IL-17A	2.0±0.6	1.7±0.2 (p=0.6272)	1.7±0.4 (p>0.9999)	1.4±0.3 (p>0.9999)
CXCL-1	165.9±27.3	205.9±20.0 (p=0.2498)	169.4±18.4 (p=0.5869)	142.2±20.2 (p=0.0824)
CXCL-2	75.0±7.7	68.0±5.7 (p=0.5039)	81.4±6.8 (p=0.4594)	73.9±5.9 (p>0.9999)

	non-defeat control	defeat control (p value*)	defeat JB-1 (p value**)	defeat sertraline (p value**)
CXCL-5	4 895.8±798.4	4 616.6±545.2 (p=0.7723)	4 545. 3±473.1 (p>0.9999)	3 623.9±471.7 (p=0.5197)
CCL-2	22.8±8.7	14.8±3.3 (p=0.3819)	6.5±0.8 (p=0.0260)	5.1±0.8 (p=0.0064)
TNF-α	6.8±3.6	1.0±0.2 (p=0.1192)	0.6±0.1 (p=0.2114)	0.5±0.1 (p=0.1521)
IFN-γ	0.7±0.3	0.7±0.12 (p=0.9739)	0.2±0.1 (p=0.1748)	0.4±0.2 (p=0.4948)

Supplementary Table S2. Serum analysis of cytokines and chemokines in the CSD experiment (mean±SEM, presented as pg/ml, n=12). *: p value calculated by unpaired t test, compared with non-defeat control. **: p value calculated by one-way ANOVA and post-hoc Bonferroni correction, compared with defeat control.

	non-defeat control	JB-1 (p value*)	sertraline (p value*)
IL-1a	614.8±72.2	802.9±245.0 (p>0.9999)	814.6±116.6 (p>0.9999)
IL-1b	18.2±10.1	6.2±0.6 (p=0.4207)	6.6±0.9 (p=0.4946)
IL-2	47.8±22.8	103.3±44.5 (p=0.6452)	33.6±2.2 (p>0.9999)
IL-4	6.5±2.6	0.9±0.2 (p=0.0570)	0.8±0.3 (p=0.0629)
IL-5	37.9±11.6	13.9±2.2 (p=0.1973)	31.8±9.6 (p>0.9999)
IL-6	50.9±12.4	21.9±4.3 (p=0.0434)	28.1±4.7 (p=0.1447)
IL-10	60.8±25.1	22.6±1.2 (p=0.1929)	16.3±2.4 (p=0.1166)
IL-12	139.2±69.7	46.0±10.2 (p=0.3427)	35.3±7.3 (p=0.3069)
IL-13	17.7±5.6	68.23±25.5 (p=0.1092)	15.8±4.9 (p>0.9999)
IL-17A	16.1±4.3	10.4±2.0 (p=0.5924)	9.2±2.2 (p=0.3680)
CXCL-1	174.9±15.6	207.1±16.9 (p=0.9819)	173.7±30.1 (p>0.9999)
CXCL-2	329.5±37.4	317.0±32.8 (p>0.9999)	363.6±28.7 (p>0.9999)

	non-defeat control	JB-1 (p value*)	sertraline (p value*)
CXCL-5	10,674.3±2,063.4	15,931.3±3,177.3 (p=0.3748)	10,856.1±1,370.2 (p>0.9999)
CCL-2	101.6±12.9	92.7±18.8 (p>0.9999)	100.7±17.2 (p>0.9999)
TNF-α	24.1±5.3	18.5±2.7 (p>0.9999)	23.2±4.9 (p>0.9999)
IFN-γ	2.9±0.8	14.6±4.9 (p=0.2279)	10.2±4.3 (p=0.7344)

Supplementary Table S3. Serum analysis of cytokines and chemokines in the non-defeat experiment (mean±SEM, presented as pg/ml, n=8). *: p value calculated by one-way ANOVA and post-hoc Bonferroni correction, compared with non-defeat control.

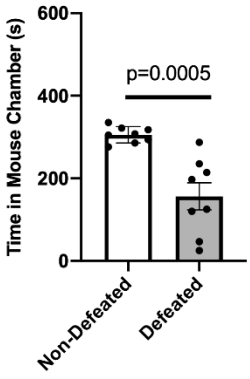

Figure S1. Time spent in social (mouse) chamber of 3-chamber sociability test, 1 day after 10-day CSD (n=8 per group).

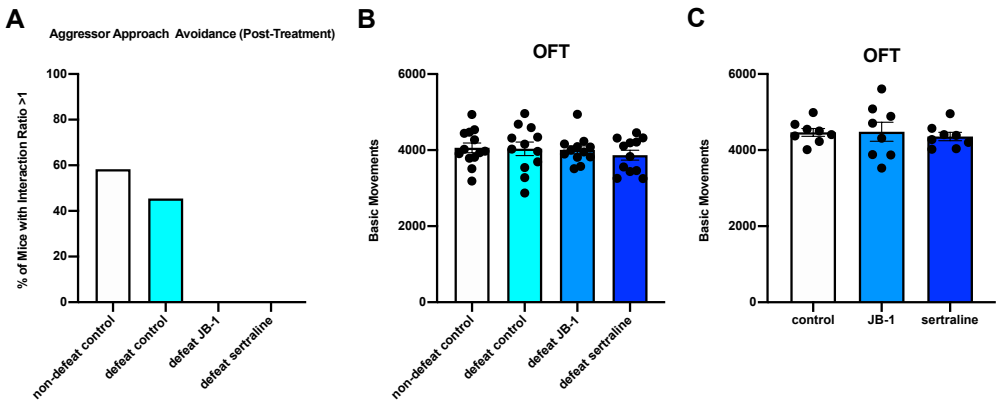

Figure S2. Supplementary behavior data.

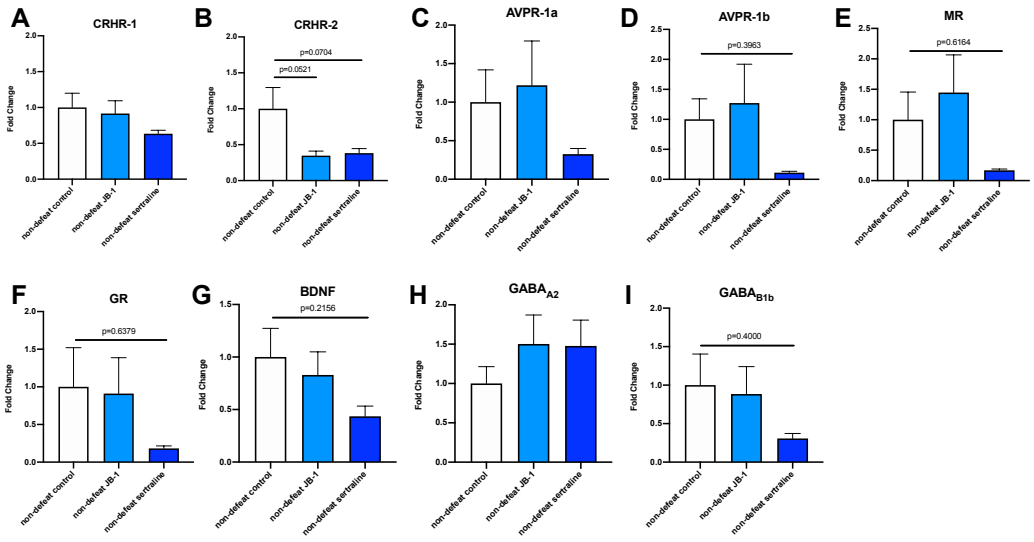

Figure S3. Stress-relate mRNA expression in hippocampus of the non-defeat experiment.

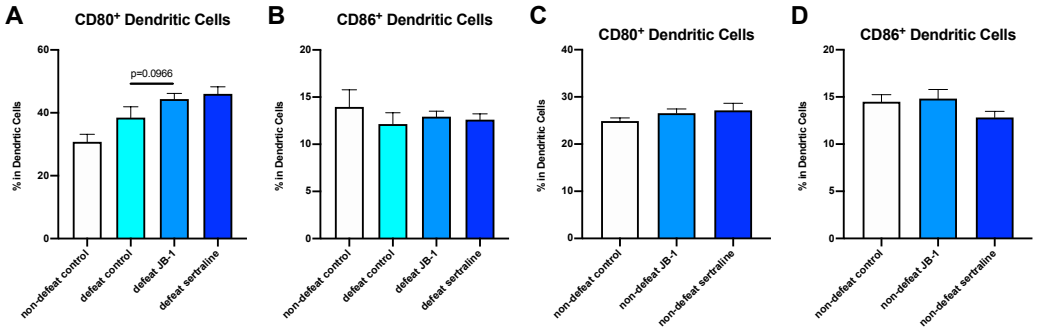

Figure S4. Flowcytometry results of CD80$^+$ and CD86$^+$ dendritic cells in splenocytes.

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CHAPTER 4.

$CD4^+CD25^+$ T Cells are Essential for Behavioral Effects of *Lactobacillus rhamnosus* JB-1 in Male BALB/c mice.

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Abstract

Over the past decade there has been increasing interest in the involvement of the microbiota-gut-brain axis in mental health. However, there are major gaps in our knowledge regarding the complex signaling systems through which gut microbes modulate the CNS. The immune system is a recognized mediator in the bidirectional communication continuously occurring between gut and brain. We previously demonstrated that *Lactobacillus rhamnosus* JB-1 (JB-1), a bacterial strain that has anxiolytic- and antidepressant-like effects in mice, modulates the immune system through induction of immunosuppressive T regulatory cells. Here we examined a potential causal relationship between JB-1 induced regulatory T cells and the observed effects on behaviour.

We found that depletion of regulatory T cells, via treatment with monoclonal antibody against CD25, inhibited the antidepressant- and anxiolytic-like effects induced by 4-week oral administration of JB-1 in mice. $Ly6C^{hi}$ monocytes were found to be decreased in JB-1 fed mice with intact regulatory T cells, but not in JB-1 fed mice following depletion. Furthermore, adoptive transfer of $CD4^{+}CD25^{+}$ cells, from JB-1 treated donor mice, but not from controls, induced antidepressant- and anxiolytic-like effects in recipient mice. $Ly6C^{hi}$ monocytes were also significantly decreased in mice receiving $CD4^{+}CD25^{+}$ cells from JB-1 fed donors.

This study identifies cells within the $CD4^{+}CD25^{+}$ population, most likely regulatory T cells, as both necessary and sufficient in JB-1-induced antidepressant- and anxiolytic-like effects in mice, providing novel mechanistic

insight into microbiota-gut-brain communication in addition to highlighting the potential for immunotherapy in psychiatric disorders.

Key Words: Probiotics; Regulatory T Cells; Depression; Anxiety; Immune System

4.1. Introduction

In any given year, approximately 1 in 5 people in North America will personally experience some form of mental health problem (Mental Health Commission of Canada, 2013). Two of the most prevalent mental health issues are anxiety disorders and major depression. These two disorders have a high comorbidity rate with occurrence of one being a risk factor for the other (Fava et al., 2000; Garber and Weersing, 2010; Jacobson and Newman, 2017). The potentially paradigm shifting implications of the microbiota-gut-brain axis have garnered much attention in recent years with suggestions it may lead to revolutionary advances in understanding etiology and pathophysiology of diseases involving mental health (Long-Smith et al., 2019). Based on existing research, microbe-based (sometimes termed 'psychobiotic') strategies have been proposed for the treatment of mood disorders including anxiety and depression. Indeed, supplementation with specific strains of bacteria has demonstrated potential benefits to mental health in several randomized controlled trials (Pirbaglou et al., 2016). However, there is still limited knowledge regarding the complex systems involved in mediating the signalling between specific gut microbes and the brain that lead to potentially beneficial effects on mood and behaviour. A better understanding of such pathways is required to fully realize the therapeutic potential of microbes in mental health (Bienenstock et al., 2015). We have previously demonstrated that oral administration of *Lactobacillus rhamnosus* JB-1 (JB-1) has anxiolytic- and antidepressant-like effects in mice with conditioned (Bharwani et al.,

2017) and trait (Bravo et al., 2011; McVey Neufeld et al., 2018) stress. In investigating the mechanism underlying the action of JB-1 on brain and behaviour, it was demonstrated that the bacteria activate the vagus nerve and that the anxiolytic- and antidepressant-like effects are prevented by sub-diaphragmatic vagotomy (Bravo et al., 2011).

Another characteristic action of JB-1 is the induction of a strong immunoregulatory response in recipient mice. Oral treatment of mice with JB-1 leads to the induction of functional regulatory T cells (Tregs), characterized by expression of CD4, CD25 and Foxp3 (Karimi et al., 2009; Karimi et al., 2012). Tregs are an important subpopulation of T cells that can drive suppression of effector T cells and other inflammatory responses (Sakaguchi et al., 2008). The attenuation of systemic inflammation by several strains of *Lactobacillus*, including JB-1, has been demonstrated to occur through induction of functional Tregs (Karimi et al., 2009; Kwon et al., 2010; Smits et al., 2005). In addition to the well described role of Tregs in suppressing inflammation, these cells have also been associated with changes in mood and behaviour in both rodent and human studies. Reduced levels of Tregs have been identified in patients with posttraumatic stress disorder (PTSD) (Sommershof et al., 2009) and depression (Li et al., 2010), while an increased proportion of $CD4^+CD25^+$ regulatory T cells have been identified in patients following antidepressant treatment (Himmerich et al., 2010). A mechanistic link between regulatory T cells and behaviour has been proposed in studies demonstrating that depletion of $CD25^+$ cells modulates behavior in mice (Kim et al.,

2012) and abolishes stress resilience associated with *M. vaccae* immunization (Reber et al., 2016); while adoptive transfer of $CD4^+CD25^+$ suppresses stress resilience induced by autoreactive T cells (Cohen et al., 2006).

Given the proposed ability of Treg to modulate behaviour (Cohen et al., 2006; Kim et al., 2012; Reber et al., 2016) and our previous observations that oral treatment with JB-1 induces production of functional regulatory cells (Karimi et al., 2009; Karimi et al., 2012), the current study utilized *in vivo* cell depletion and adoptive transfer to provide evidence of a role for Treg in mediating anxiolytic- and antidepressant-like effects of the psychoactive bacteria.

4.2. Methods

4.2.1. Animals. Male BALB/c mice, 6-8 weeks old were acquired from Charles River (Montreal, QC, Canada) and allowed to acclimatise to the housing facility for ⩾7 d before experiments began. BALB/c mice were used as they are considered to be a high trait anxiety or 'anxiety-prone' strain (McVey Neufeld et al., 2018; Sartori et al., 2011; Savignac et al., 2011) and baseline behaviours of this strain are sensitive to modulation by JB-1 (Bravo et al., 2011; McVey Neufeld et al., 2018). Mice were housed 3 per cage, at 25 ℃ on a 12h light/dark cycle (5am to 5pm), with access to regular chow and water ad libitum. All experimental procedures were carried out in accordance with Canadian Council on Animal Care guidelines and were approved by the McMaster Animal Research Ethics Board.

4.2.2. Probiotic Administration. *Lactobacillus rhamnosus* JB-1 was gifted from Alimentary Health Ltd., Cork, Ireland (McVey Neufeld et al., 2018). The bacteria were delivered in drinking water via a 50 ml conical tube with fitted sipper at $1x10^9$ CFU per day based on a previously quantified average daily water consumption of 3 ml per mouse. Treatment was administered for 28 days prior to behavioural testing and continued throughout the testing period for a total of 39 days treatment. Water bottles containing JB-1 were prepared freshly every day. Mice in the control group were given drinking water alone.

4.2.3. Depletion of $CD4^+CD25^+$ Cells. Anti-CD25 monoclonal antibody (clone: PC61-61.5.3, BioXCell, NH, USA). The antibody (1mg in 125 μl saline) was injected intraperitoneally the same day as JB-1 treatment began, with a repeat injection 19 days later to ensure continued depletion, since we found the antibody could be valid for depletion for 3 weeks (data not shown). The control group received corresponding injections of 1 mg Isotype IgG (clone: HRPN, BioXCell, NH, USA) in 125 μl saline. The efficacy of Treg depletion was tested by flow cytometry after sacrifice. The timeline of depletion experiments is illustrated in Fig. 1A.

4.2.4. Behavioral Testing. For each behavior test, mice were brought from the colony room into a separate behavior room and allowed 1 hour of habituation. All behavioural equipment was thoroughly cleaned with distilled water and dried

between animals tested. All behavior tests were conducted during the time period from 9am to 11am in the light cycle.

Tail Suspension Test. Mice were suspended by the tail using lab tape measured to 17 cm (2 cm for taping, 15 cm for hanging) such that they could not escape or touch any surface. The test continued for 6 minutes and was video recorded. The video was analysed by a blinded researcher, and percentage of freezing was calculated (McVey Neufeld et al., 2018).

Elevated Plus Maze. We used a black Plexiglas plus sign apparatus, with two open arms (30×5 cm), two closed arms (30×5×15cm) and a central platform (5×5cm), elevated 60 cm above the floor. After 1-hour habituation in the room, mice were placed onto the central platform facing an open arm and allowed a 5-minute exploration. Entry into the arms was defined as all four paws entering an arm. The data were recorded by Motor Monitor Software (Kinder Scientific, Poway, Canada) (Bravo et al., 2011).

Open Field Test. Mice were placed in a clear Plexiglas enclosure (18×38 cm) separately for 30 minutes and allowed to freely explore. General locomotion and time spent in the center were recorded by Motor Monitor Software (Kinder Scientific) (Bravo et al., 2011).

Light-Dark Test. Light-dark test was performed using the same enclosure as for the open field test, modified by addition of a black Plexiglas box separating the field into a light half and dark half. An open doorway connecting the two zones allowed the animal to enter both zones freely. Animals were placed in the light zone and

allowed to freely explore for 10 minutes. Time spent in each zone was recorded by Motor Monitor Software (Kinder Scientific) (Bharwani et al., 2017).

4.2.5. Magnetic-activated Cell Sorting and Adoptive Transfer of $CD4^+CD25^+$ Cells.

Donor mice were fed either water or JB-1 for 2 weeks prior to tissue harvest and preparation of a single cell solution of splenocytes. This treatment period is enough for JB-1 to induce systemic functional Tregs (Karimi et al., 2009). $CD4^+CD25^+$ cells were isolated by Dynabeads isolation kit (ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. $CD4^+$ T cells were isolated by negative selection, and then $CD4^+CD25^+$ cells were isolated by positive selection. The purity of $CD4^+CD25^+$ cells was tested by flow cytometry. Approximately $1x10^6$ $CD4^+CD25^+$ cells in 500-700 µl phosphate-buffered saline (PBS) solution was injected through the tail vein to each JB-1 naïve recipient mouse, and combined cells from 3 donors was sufficient to transfer into 2 recipients. An additional control group of mice was injected with PBS only. The timeline of adoptive transfer experiments is shown in Figure 4.

4.2.6. Flow Cytometry. A single cell suspension of splenocytes was prepared following tissue harvest and lysis of red blood cells. Splenocytes ($1x10^6$ per well in 96-well plate) were stained first with extracellular antibodies, then cytofix (Invitrogen™, ThermoFisher Scientific) was applied followed by staining with

intracellular antibodies if required. The following panels of conjugated antibodies were used for staining: Tregs (CD3-APC-eFluor780, CD4-FITC, CD25-APC, Foxp3-PE), $CD11b^+$ cells (CD11b-PE, Ly6C-APC, CCR2-FITC). For Th1/Th2 identification, splenocytes were stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin, then stained by IFN-γ-APC and IL-4-PerCP-eFluor710. All conjugated antibodies were purchased from Invitrogen™, ThermoFisher Scientific, and diluted to 1:200. Flow cytometry was conducted using BD FACSCelesta™ (BD Biosciences, San Jose, CA, USA) and results analysed by FlowJo software (FlowJo LLC, Ashland, OR, USA), with doublets and cell debris excluded by forward scatter (FSC) and side scatter (SSC) gating. Representative dot plots of each group were shown in Fig. S1A-E and S2.

4.2.7. Statistical Analysis. Data were analysed using the software GraphPad Prism 8.0 (Graphpad Software, San Diego, CA, USA). For the $CD4^+CD25^+$ cell depletion experiment (2 interventions, 4 groups), two-way ANOVA and Tukey's multiple comparison test was used. For the results of recipient mice in the $CD4^+CD25^+$ cells adoptive transfer experiment (1 intervention, 3 groups), one-way ANOVA with Fisher's LSD test was used. For comparisons of donor mice, parametric t test was used. Results shown in the figures were illustrated as mean ± standard error of the mean (SEM) if applicable. *p* value smaller than 0.05 was considered as statistically significant.

4.3. Results

4.3.1. Anti-CD25 mAb treatment successfully depleted $CD4^+CD25^+Foxp3^+$ T cells.

According to flow cytometry results of splenocytes, JB-1 treatment led to a significant increase of the proportion of $CD4^+CD25^+Foxp3^+$ T cells in mice receiving control isotype IgG group (Isotype IgG/Water: 11.6±0.4%, Isotype IgG/JB-1: 14.6±0.4%, $p<0.0001$), indicating Treg induction as previously described (Karimi et al., 2012). The groups treated with anti-CD25 mAb showed very low level of $CD4^+CD25^+$ cells (Anti-CD25/Water: 0.7±0.1%, Anti-CD25/JB-1: 0.9±0.1%, Fig. 1B), indicating successful depletion of this cell population.

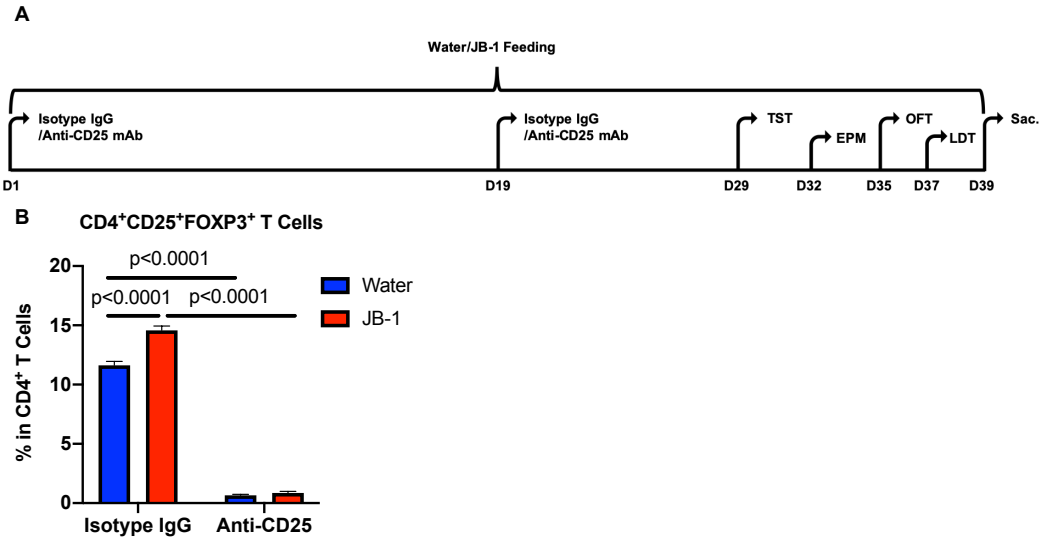

Figure 1. (A) Timeline of the experiment of $CD4^+CD25^+$ T cell depletion and JB-1 administration. TST: tail suspension test. EPM: elevated plus maze. OFT: open

field test. LDT: Light-dark test. **(B)** Anti-CD25 mAb successfully depleted $CD4^+CD25^+Foxp3^+$ T cells (n=6 in each group) in spleen.

4.3.2. Anti-CD25 mAb treatment reduced the behavioral effects of L. rhamnosus JB-1.

Tail suspension test (TST) was utilised to assess learned helplessness, an important component of depressive-like behaviour in rodents. Anti-CD25 mAb alone did not affect immobility time significantly (Isotype IgG/Water: 24.0±0.4%, Anti-CD25/Water: 20.1±3.3%, p=0.8240). We demonstrated, as previously reported (McVey Neufeld et al., 2018) that JB-1 treatment reduced immobility time compared to water fed controls (Isotype IgG/JB-1: 11.7±2.5%, p=0.0399). However, JB-1 did not significantly alter immobility time in mice depleted of $CD4^+CD25^+$ cells through treatment with anti-CD25 mAb (Anti-CD25/JB-1: 23.4±2.5%, p=0.8706, Fig. 2A).

Elevated plus maze (EPM), light dark test (LDT) and the open field test (OFT) were used to test for anxiety-like behaviors. In the EPM, JB-1 significantly increased time spent in open arm in the isotype IgG injected groups (Isotype IgG/Water: 10.0±2.5s, Isotype IgG/JB-1: 25.2±4.1s, p=0.0014). As observed in the TST, JB-1's behavioural effects were nullified in the anti-CD25 mAb treated groups (Anti-CD25/Water: 7.8±1.8s, Anti-CD25/JB-1: 5.3±1.4s, p=0.9198, Fig. 2B). In the OFT, no significant difference in general locomotor activity was observed between all four groups (Fig. 2C), and no significant difference was found in time spent in

center zone (Fig. 2D). In the LDT, no significant result was found between groups (Fig. 2E).

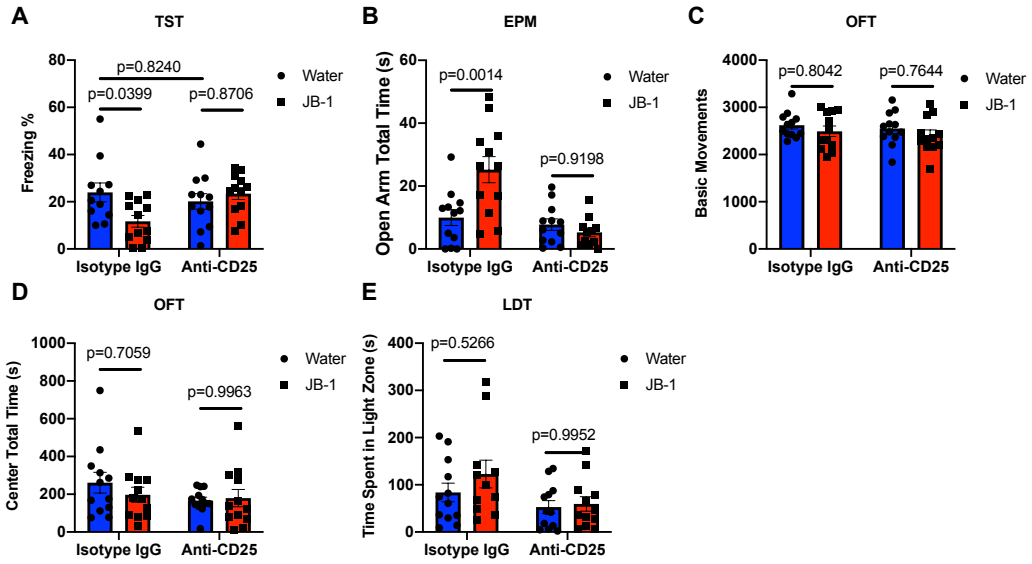

Figure 2. CD4^{+}CD25^{+} T cell depletion inhibited the antidepressant-like and anxiolytic effects induced by JB-1. (A) Percentage of freezing time in tail suspension test (n=11-12 in each group). **(B)** Time spent in open arm in elevated plus maze (n=11-12 in each group). **(C)** Basic movements (general locomotor activity) in open field test (n=12 in each group). **(D)** Time spent in center zone in open field test (n=12 in each group). **(E)** Time spent in light zone in light-dark test (n=12 in each group).

4.3.3. JB-1 led to decreased Ly6C^{hi} monocytes which was not observed following anti-CD25 mAb treatment.

Ly6C^{hi} monocytes are an important inflammatory signal from periphery to brain which is correlated with behavioral changes (Wohleb et al., 2014). We found that JB-1 feeding, in the isotype IgG control mice, led to a significant decrease in Ly6C^{hi} monocytes, compared to isotype IgG and water treated group (Isotype IgG/Water: 4.7±0.4%, Isotype IgG/JB-1: 3.2±0.5%, p=0.0358). The difference in Ly6C^{hi} monocytes following JB-1 exposure was not observed in mice treated with anti-CD25 mAb treatment (Anti-CD25/Water: 2.3±0.4%, Anti-CD25/JB-1: 3.1±0.2%, p=0.3810). However, anti-CD25 treatment alone caused a significant decrease of Ly6C^{hi} monocytes in the anti-CD25 and water fed group, compared to isotype IgG and water fed group (p=0.0007, Fig. 3A).

T helper 1 (Th1) and T helper 2 (Th2) cells are effector CD4 T cell populations usually involved in pro-inflammatory effects and are supressed by Tregs. In water fed mice, anti-CD25 mAb led to significant increases in both Th1 and Th2 cells (Th1: Isotype IgG/Water: 2.4±0.2%, Anti-CD25/Water: 4.4±0.6%, p=0.0151; Th2: Isotype IgG/Water: 2.9±0.2%, Anti-CD25/Water: 4.1±0.3%, p=0.0064). However, in JB-1 fed mice, Anti-CD25 mAb treatment did not alter the proportion of Th1 or Th2 cells (Th1: Isotype IgG/JB-1: 2.3±0.3%, Anti-CD25/JB-1: 3.4±0.4%, p=0.3254; Th2: Isotype IgG/JB-1: 2.9±0.3%, Anti-CD25/JB-1: 3.6±0.1%, p=0.2563, Fig. 3B & C). Dendritic cell activation was also assessed but no significant difference was found between all treatment groups in the proportion of dendritic cells expressing the activation markers CD80^{+} or CD86^{+} (Fig. 3D & E).

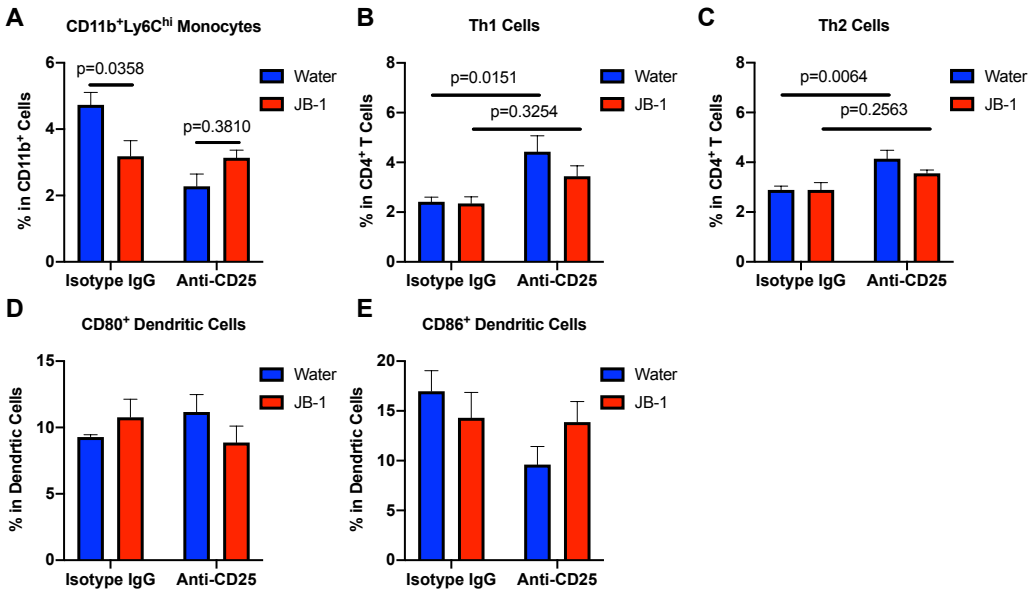

Figure 3. Changes of spleen immune cells after Treg depletion in JB-1 fed mice (n=5-6 in each group). (A) $CD11b^{+}Ly6C^{hi}$ monocytes. **(B)** $CD4^{+}IFN\text{-}\gamma^{+}IL\text{-}4^{-}$ cells (Th1 cells). **(C)** $CD4^{+}IFN\text{-}\gamma^{-}IL\text{-}4^{+}$ cells (Th2 cells). **(D)** $CD11c^{+}MHCII^{+}CD80^{+}$ dendritic cells. **(E)** $CD11c^{+}MHCII^{+}CD86^{+}$ dendritic cells.

4.3.4. Adoptive transfer of CD4+CD25+ cells from JB-1 fed donors had anxiolytic- and antidepressant-like effects on recipient mice.

One week after receiving cell transfer recipient mice were assessed in the same series of behavior tests used in depletion experiments (timeline illustrated in Fig. 4). No significant difference was found in any comparisons between the group with PBS injection and the group with $CD4^{+}CD25^{+}$ cells adoptively transferred from water fed group. The statistical comparisons specifically reported below are thus

between the two groups with $CD4^+CD25^+$ cells transferred from water- or JB-1 fed groups.

TST showed a significant reduction in freezing time for mice receiving $CD4^+CD25^+$ cells from JB-1 fed donors, compared to those that received $CD4^+CD25^+$ cells from water fed donors ($CD4^+CD25^+$ cells-Water:30.1±2.8%, $CD4^+CD25^+$ cells-JB-1: 21.8±2.9%, p=0.0463, Fig. 5A). EPM demonstrated that mice with $CD4^+CD25^+$ cells transferred from JB-1 fed donors spent significantly more time in the open arm ($CD4^+CD25^+$ cells-Water: 23.8±4.0s, $CD4^+CD25^+$ cells-JB-1: 63.5±5.5s, p<0.0001, Fig. 5B). We also found that according to OFT results, mice who received $CD4^+CD25^+$ cells from JB-1 fed donors spent significantly greater time in the center area of the OFT ($CD4^+CD25^+$ cells-Water: 162.2±34.6s, $CD4^+CD25^+$ cells-JB-1: 276.3±42.3s, p=0.0496, Fig. 5C). There was no difference between groups in the LDT (Fig. 5D).

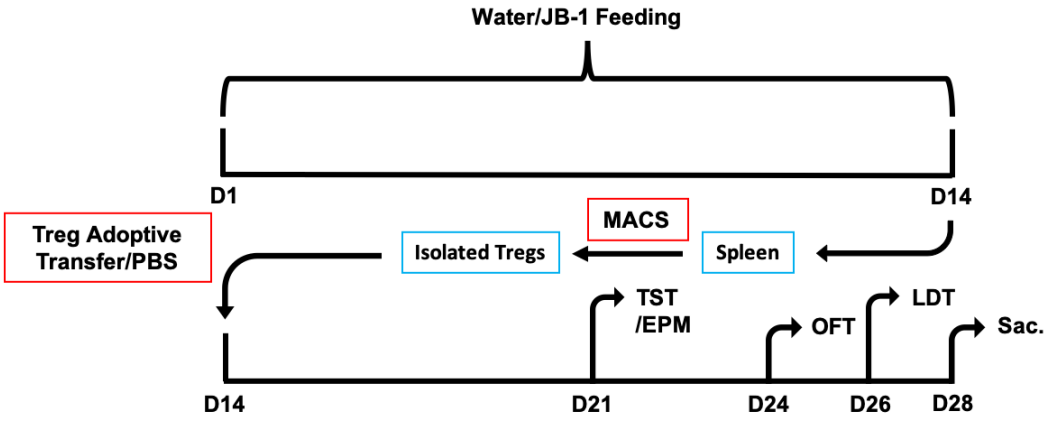

Figure 4. Timeline of the experiment of $CD4^+CD25^+$ cell adoptive transfer from JB-1 fed donors into recipient mice.

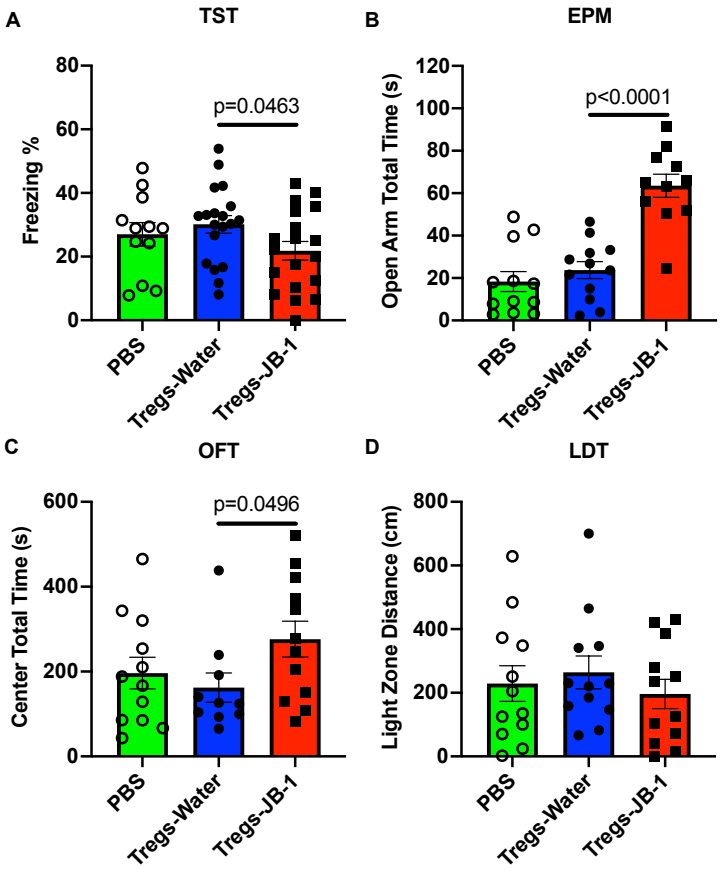

Figure 5. **Adoptive transfer of $CD4^+CD25^+$ cells from JB-1 fed donors induce antidepressant-like and anxiolytic effects in recipient mice. (A)** Percentage of freezing time in tail suspension test (n=12 in PBS group, n=19 in both $CD4^+CD25^+$ cells-Water and $CD4^+CD25^+$ cells-JB-1 group). **(B)** Time spent in open arm in elevated plus maze (n=11-12 in each group). **(C)** Time spent in center zone in open field test (n=10-12 in each group). **(D)** Time spent in light zone in light-dark test (n=12 in each group).

4.3.5. $CD4^+CD25^+$ cells from JB-1 fed donor mice reduced $Ly6C^{hi}$ monocytes in recipient mice.

The purity of the $CD4^+CD25^+$ cells isolated from donors was approximately 89%, as illustrated in Fig. 6A. In recipient mice, we found a significant increase in both groups receiving cell transfer, compared to the PBS injected group (PBS: 10.1±0.6%, $CD4^+CD25^+$ cells-Water: 12.6±0.2%, p=0.0017, $CD4^+CD25^+$ cells-JB-1: 12.8±0.4%, p=0.0009, Fig. 6B). The expression of Foxp3 was also increased in both groups received $CD4^+CD25^+$ (PBS: 9.3±0.5%, $CD4^+CD25^+$ cells-Water: 12.0±0.1%, p=0.0005, $CD4^+CD25^+$ cells-JB-1: 12.0±0.5%, p=0.0005, Fig. 6C).

We then assessed monocytes in recipient mice and found a significant decrease in the $Ly6C^{hi}$ monocytes population in JB-1-$CD4^+CD25^+$ cells recipient ($CD4^+CD25^+$ cells-Water: 4.8±0.7%, $CD4^+CD25^+$ cells-JB-1: 3.0±0.2%, p=0.0118, Fig. 6D). We also assessed expression of C-C chemokine receptor 2 (CCR-2), as an index of chemotactic capacity of $CD11b^+$ cells. There was a significant decrease in the proportion of cells expressing CCR-2 in mice receiving cells from the JB-1 fed donors ($CD4^+CD25^+$ cells-Water: 23.8±1.7%, $CD4^+CD25^+$ cells-JB-1: 18.1±1.0%, p=0.0281 with $CD4^+CD25^+$ cells-Water group, Fig. 6E). Moreover, no significant difference was found in either Th1 or Th2 cells amongst three recipient groups (Fig. 6F and 6G).

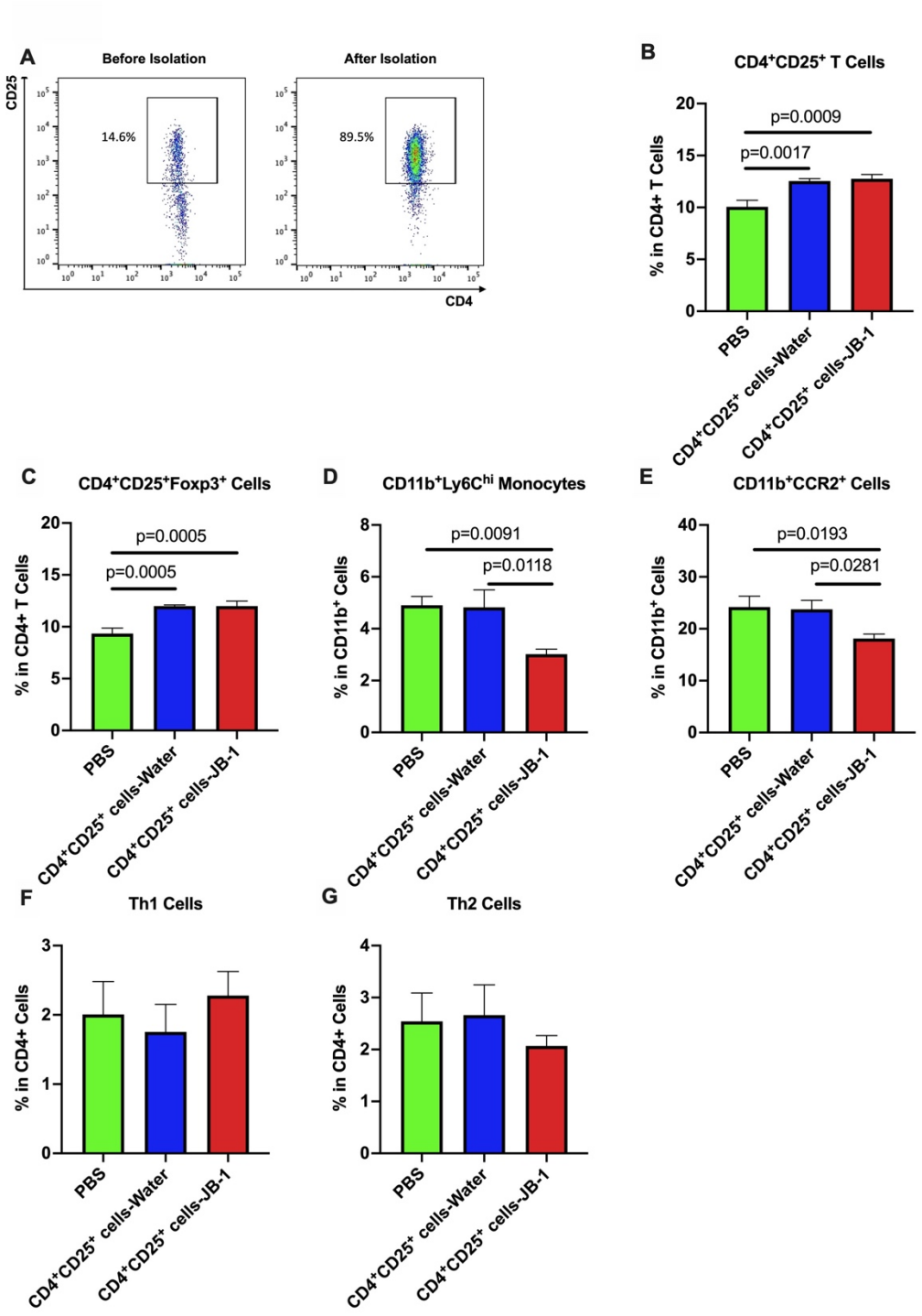
A
Before Isolation
After Isolation
CD25
CD4
14.6%
89.5%
B
CD4+CD25+ T Cells
% in CD4+ T Cells
p=0.0009
p=0.0017
C
CD4+CD25+Foxp3+ Cells
p=0.0005
p=0.0005
D
CD11b+Ly6Chi Monocytes
% in CD11b+ Cells
p=0.0091
p=0.0118
E
CD11b+CCR2+ Cells
p=0.0193
p=0.0281
F
Th1 Cells
% in CD4+ Cells
G
Th2 Cells
PBS
CD4+CD25+ cells-Water
CD4+CD25+ cells-JB-1

Figure 6. Changes of spleen immune cells after $CD4^+CD25^+$ T cells adoptive transfer. (A) Illustration of the purity of Tregs after magnetic-activated cell sorting. **(B)** $CD4^+CD25^+$ cells of recipient mice (n=6 in each group). **(C)** $CD4^+CD25^+Foxp3^+$ cells of recipient mice (n=6 in each group). **(D)** $Ly6C^{hi}$ monocytes in recipient mice (n=6 in each group). **(E)** $CD11b^+CCR2^+$ cells in recipient mice (n=6 in each group). (**F**) Th1 cells in recipient mice (n=6 in each group). (**G**) Th2 cells in recipient mice (n=6 in each group).

4.4. Discussion

Our study provides evidence supportive of a crucial role for $CD4^+CD25^+$ regulatory T cells in mediating at least some of the anxiolytic- and antidepressant-like effects of the psychoactive bacteria *L. rhamnosus* JB-1. Depletion of $CD4^+CD25^+$ cells prevented the effects of JB-1 on behavior while adoptive transfer of $CD4^+CD25^+$ cells isolated from JB-1 treated mice could replicate the behavioral effects of JB-1 feeding in recipient mice.

There is good evidence linking the immune system to mood and behaviour. Both depression and anxiety have been associated with immunoregulatory dysfunction and a shift towards an inflammatory immune profile (Irwin and Miller, 2007; Liukkonen et al., 2011; O'Donovan et al., 2010). In mice, chronic social defeat, leads to anxiety and depressive-like symptoms with an associated increase in serum IL-6, and reduction in Tregs (Bharwani et al., 2016). A causal relationship between inflammation and mood disorders is supported by observations that

administration of pro-inflammatory cytokines to non-depressed subjects leads to symptoms of depression (Miller and Raison, 2016), while, in animal models, activation of the innate immune response has been shown to lead to development of symptoms associated with mood and cognitive disorders (Dantzer et al., 2008; Godbout et al., 2008; Miller, 2010; Raison et al., 2006; Wilson et al., 2002). Moreover, meta-analysis, suggests that anti-inflammatory treatment, particularly celecoxib, a COX-2 selective nonsteroidal anti-inflammatory drug, can decrease symptoms of depression (Köhler et al., 2014). Thus, accumulated evidence suggests that the maintenance of immune balance plays a role in protecting against the development of anxiety and depression.

Regulatory T cells are a population of $CD4^+$ T cells which have immunosuppressive effects. Characterised by the expression of CD25 and Foxp3, the normal function of Tregs include maintaining immune tolerance to self-antigens, and preventing autoimmune/autoinflammatory disease (Plitas and Rudensky, 2016).

Tregs have also been associated with changes in mood and behaviour. Li et al. reported reduced levels of Tregs in peripheral blood in patients with major depressive disorder compared to healthy controls (Li et al., 2010) and reduced populations of regulatory T cells have also been demonstrated in PTSD (Sommershof et al., 2009).

In animal models, depletion of Tregs with anti-CD25 mAb prevented the protective effect of immunization with *M. vaccae* on the development of anxiety-

like behaviour following chronic social stress in mice (Reber et al., 2016). Furthermore, Kim et al. found that anti-CD25 mAb treatment led to decreased time spent in open arm of the EPM and increased immobility time in the forced swim test in non-stressed C57BL/6 mice, which they attributed to Treg modulation of anxiety- and depressive-like behaviour (Kim et al., 2012). While both studies suggest the necessity of Tregs in attenuating anxiety- and depressive-like behaviours, ours is the first to provide evidence, via adoptive transfer, that changes in Treg activity may also be sufficient to modulate these behaviours.

Of note, unlike Kim et al. (Kim et al., 2012) we did not see changes in baseline behaviour of our mice following anti-CD25 mAb treatment. This may be attributable to differences between the behavioral phenotype and/or immune profile of the mouse strains used. BALB/c trait behaviours are considered as being “anxiety-prone” or “highly anxious” compared to other strains of mice including C57BL/6 and Swiss Webster (Sartori et al., 2011; Savignac et al., 2011). Correspondingly, the normal behaviours of BALC/c mice are sensitive to modulation by JB-1 (Bravo et al., 2011; McVey Neufeld et al., 2018) while those of C57BL/6 and Swiss Webster mice are not (Bharwani et al., 2017; McVey Neufeld et al., 2018). With relation to the immune system, BALB/c mice have a “Th2 bias” (Hsieh et al., 1995) and effector T cells from this strain are more responsive to suppression by Tregs than those from C57BL/6 mice (Chen et al., 2005).

A limitation to the approach of targeting CD25 expressing cells in both depletion and adoptive transfer experiments is that this marker is not specific for

regulatory T cells. While almost 80% of cells in the $CD4^+CD25^+$ population express Foxp3 (Fig. S1F), marking them as Tregs, the $CD4^+CD25^+$ population will also contain effector T cells, cells in an intermediate stage of differentiation into Tregs (Josefowicz et al., 2012) and, potentially, Foxp3 negative regulatory populations (Niedbala et al., 2013). Similarly, due to constraints of the isolation method adoptively transferred cells are not a pure $CD4^+CD25^+$ population and do contain other cell types. Based on these limitations we cannot definitively identify the immune component driving JB-1 effects behavior, but Tregs remain the most likely candidate. The induction of functional Tregs by JB-1 and other *Lactobacillus* strains has been well described (Karimi et al., 2009; Karimi et al., 2012; Kim et al., 2014; Kwon et al., 2010). We have previously demonstrated that, when given orally, JB-1 is taken up by dendritic cells in the Peyer's patches, and that the dendritic cells containing JB-1 then take on a tolerogenic phenotype and can induce Tregs production (Karimi et al., 2012). Importantly, $CD4^+CD25^+$ cells isolated from JB-1 fed mice have an enhanced ability to suppress effector T cell function and when adoptively transferred can suppress allergic airway inflammation, an effect that does not occur with transfer of cells from water fed mice (Karimi et al., 2009). In the current study, we again demonstrate modified function of $CD4^+CD25^+$ cells from JB-1 as adoptive transfer of cells from mice treated with the bacteria, but not water fed controls, could reduce anxiety- and depressive-like behaviours.

How JB-1 induced Tregs mediate changes in the CNS leading to modifications in behaviour was not explored in the current study. However, the fact

that direct JB-1 feeding and adoptive transfer of JB-1 induced Tregs decreased $Ly6C^{hi}$ monocytes in our study may be significant. Activated monocytes have been identified as important communicators between the peripheral immune system and the central nervous system. Wohleb et al. demonstrated that repeated social defeat induces an increase in circulating $Ly6C^{hi}$ monocytes, and recruitment of these monocytes to the brain serves to amplify neuro-inflammation in response to stress and promote anxiety-like behavior (Wohleb et al., 2013). Furthermore, a high level of $Ly6C^{hi}$ monocytes has been reported as an indicator of stress susceptibility in mice (Gururajan et al., 2019). Here we provide evidence that JB-1 induced functional changes in $CD4^{+}CD25^{+}$ cells attenuate $Ly6C^{hi}$ monocytes levels. However, depletion of Tregs with anti-CD25 mAb itself caused a decrease in $Ly6C^{hi}$ monocytes levels in the spleen, an effect we cannot currently explain and which suggests the relationship between these cell populations may be complex. Future studies investigating downstream mechanisms of how the JB-1 effects on immune function are translated to the central nervous system and any potential role for $Ly6C^{hi}$ monocytes are clearly warranted.

An issue that remains to be addressed is how to reconcile the crucial role for Tregs in mediating the behavioral effects of JB-1 with our previous evidence indicating a vagus nerve dependent mechanism. It may be that parallel neural and immune pathways are required to induce changes in the brain that result in a reduction in anxiety- and depressive-like behaviours. There is a close relationship between the vagus nerve and immune system, with the anti-inflammatory action of

the vagus nerve now well established. Sub-diaphragmatic vagotomy has been demonstrated to enhance proliferation and pro-inflammatory cytokine release from effector T cells (Karimi et al., 2010) and decrease the population of Tregs in the spleen (O'Mahony et al., 2009). This opens the possibility that the attenuation of effects of JB-1 on behaviour in vagotomised mice could be due to the loss of efferent immunoregulatory signals leading to inhibition or masking of the suppressive effect of JB-1 induced Tregs, rather than to a loss of direct afferent gut-brain signaling. Novel insights into gut-brain signalling will likely emerge from future detailed studies of the relationship between the vagus nerve and immune system in relation to CNS effects.

In conclusion, our study indicates that $CD4^+CD25^+$ cells, most likely regulatory T cells, are both necessary and sufficient for *L. rhamnosus* JB-1 induced antidepressant and anxiolytic-like effects on the trait behaviour of male BALB/c mice. Although the downstream mechanism is unclear, a Treg driven reduction in activated monocytes may be involved. This work provides novel insight into how specific organisms in the gut can communicate with the brain and further highlights the importance of the immune system in modulating behavior as well as the potential for immunotherapy in the management of psychiatric disorders.

Conflict of interests

The authors declare no competing interests.

Contributors

Y.L. and P.F., designed the experiments. Y.L. and K.-A.M.N. performed experiments. Y.L., M.F.M. and K.-A.M.N. analysed the data and constructed the figures. Y.L. and P.F. wrote and edited the manuscript, which was reviewed before submission by all authors.

Acknowledgements

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4.5. Supplementary Information

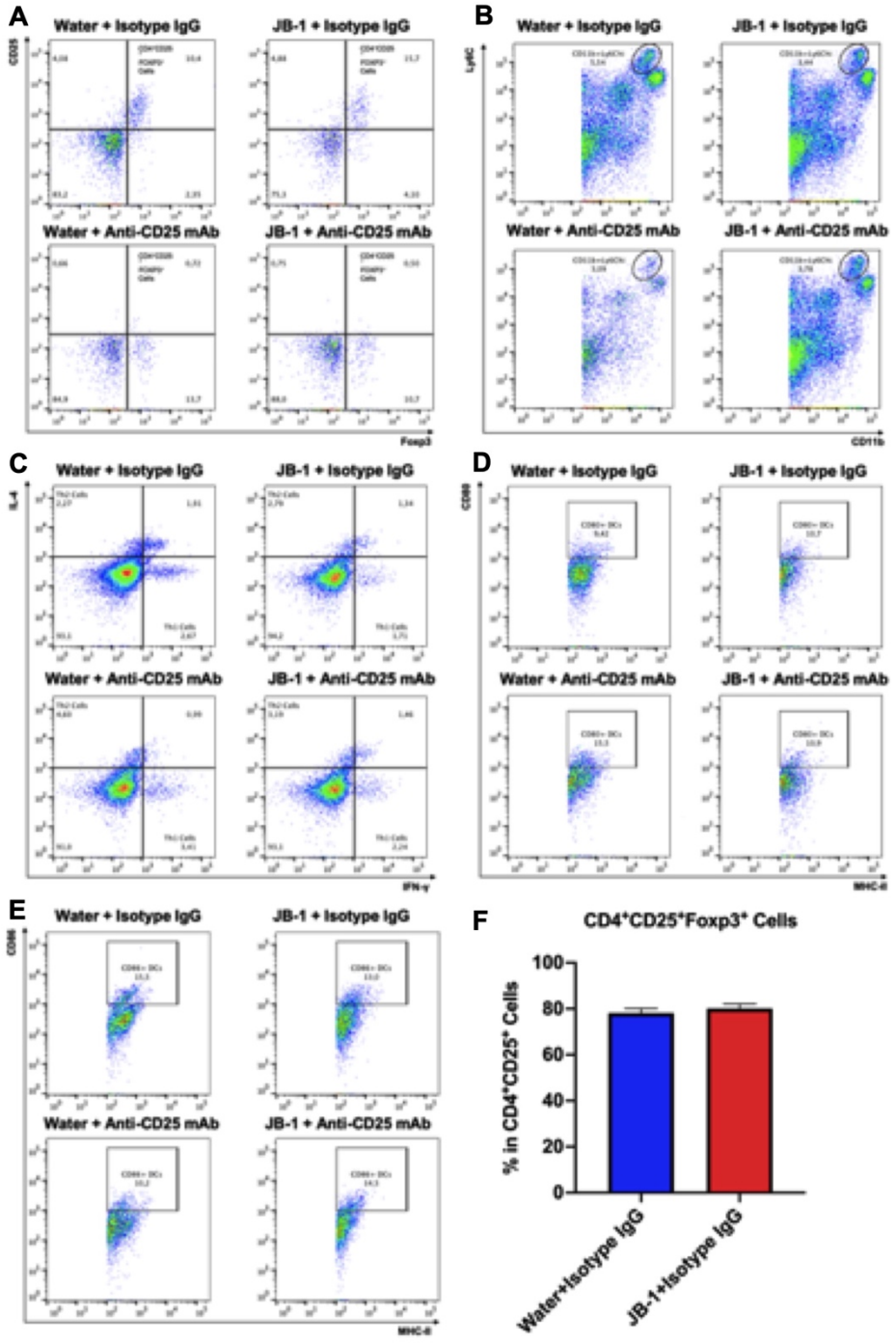

Figure S1. Flow cytometry results in $CD4^+CD25^+$ cell depletion experiment. **(A-E)** Representative dot plots of flow cytometry. **(A)** Percentage of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+$ T cells. **(B)** $Ly6C^{hi}$ monocytes. **(C)** Percentage of Th1 and Th2 cells in $CD4^+$ T cells. **(D)** Percentage of $CD80^+$ dendritic cells (DCs) in $CD11c^+$MHC II^+ cells. **(E)** Percentage of $CD86^+$ dendritic cells (DCs) in $CD11c^+$MHC II^+ cells. **(F)** Percentage of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+CD25^+$ cells of two groups injected with isotype IgG.

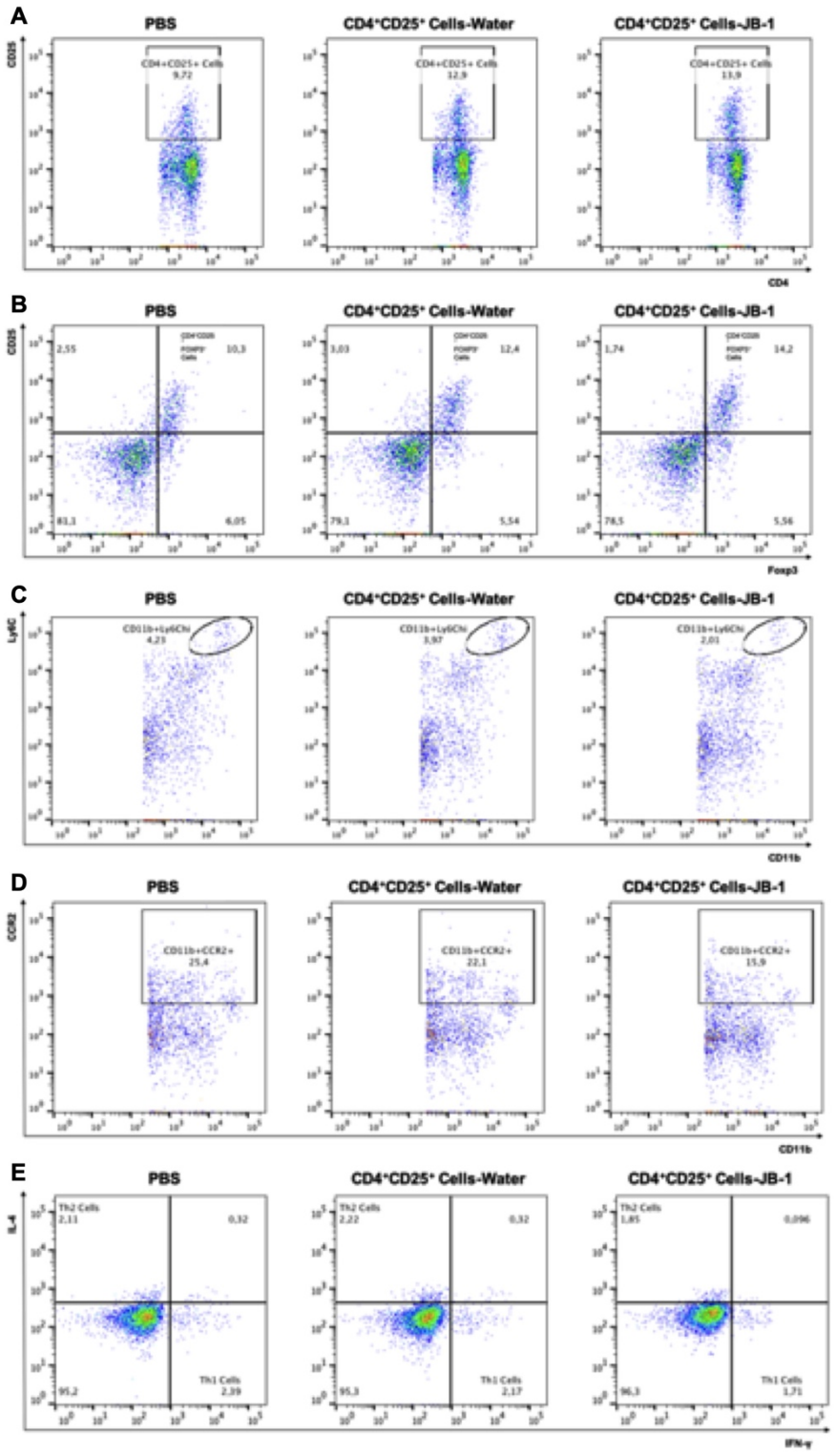
A
PBS
CD4+CD25+ Cells-Water
CD4+CD25+ Cells-JB-1
CD25
CD4
B
PBS
CD4+CD25+ Cells-Water
CD4+CD25+ Cells-JB-1
CD25
Foxp3
C
PBS
CD4+CD25+ Cells-Water
CD4+CD25+ Cells-JB-1
Ly6C
CD11b
D
PBS
CD4+CD25+ Cells-Water
CD4+CD25+ Cells-JB-1
CCR2
CD11b
E
PBS
CD4+CD25+ Cells-Water
CD4+CD25+ Cells-JB-1
IL-4
IFN-γ

Figure S2. Representative dot plots of flow cytometry in $CD4^+CD25^+$ cell adoptive transfer experiment. (A) Percentage of $CD4^+CD25^+$ cells in $CD4^+$ T cells. **(B)** Percentage of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+$ T cells. **(C)** $Ly6C^{hi}$ monocytes. **(D)** $CCR2^+$ cells in $CD11b^+$ cells. **(E)** Percentage of Th1 and Th2 cells in $CD4^+$ T cells.

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CHAPTER 5

Loss of vagal integrity disrupts immune and endocrine components of the microbiota-gut-brain axis.

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Abstract

Previous studies have demonstrated that the vagus nerve is essential to the anxiolytic effects conducted by ingestion of the bacteria, *Lactobacillus rhamnosus* JB-1 on mice. Microglia are known as resident immune cells in brain. By arousing neuroinflammation, microglia are involved in the formation of altered behaviors after stress. The purposes of the present study were to determine if the beneficial effects of JB-1 on behaviors were associated with limitation of microglial activation in the hippocampus of mice, and whether these effects depended on the integrity of vagus nerve. Male adult BALB/c mice were treated with vagotomy or sham surgery, followed by chronic administration of JB-1 for longer than 14 days. Reduced activated microglia were found in hippocampus of sham operated mice fed with JB-1, compared to sham mice fed with water, which was correlated with ameliorated anxiety-like behaviors, as indicated by open field test and elevated plus maze. These JB-1 induced effects were not seen in vagotomized groups. Moreover, we also found reduced activation of hypothalamic-pituitary-adrenal axis (HPA axis), indicated by plasma corticosterone after acute restraint stress, in the sham group fed by JB-1, which was also not observed following vagotomy. These novel findings suggest an important role of vagus nerve in mediating central immunosuppressive functions and endocrine response to maintain behavioral homeostasis in mice.

Key Words: Vagus Nerve; Microglia; HPA Axis; Stress; Probiotics

5.1. Introduction

It is now widely acknowledged that the microbiota-gut-brain axis plays an essential role in maintaining homeostasis, and that imbalance within the axis can lead to psychiatric disorders (Forsythe, Kunze, & Bienenstock, 2016). Numerous studies in animal models have identified that alteration of the microbiota or exposure of the gut to specific bacteria can modulate brain chemistry, stress responses and anxiety/depressive-like behaviors.

Oral treatment with *Lactobacillus rhamnosus* JB-1 has been demonstrated to have anxiolytic effect in mice (Bravo et al., 2011) and to prevent social defeat induced changes in behaviour (Bharwani, Mian, Surette, Bienenstock, & Forsythe, 2017). *L. rhamnosus* JB-1 also reduces plasma corticosterone levels following acute restraint stress indicating that HPA response is modulated by this probiotic treatment (McVey Neufeld, Kay, & Bienenstock, 2018).

The vagus nerve is a component of parasympathetic nervous system and a major hardwired connection between the brain and gastrointestinal tract. Previous studies have identified that the anxiolytic and antidepressant-like effects of JB-1 depend on an intact vagus nerve connection between the gut and the brain (Bravo et al., 2011). However, the exact relationship between gut-brain signaling along the vagus and the effects of gut bacteria on brain function and behaviour is unclear. In particular it is not known how the vagus nerve is related to immune signaling that also appears to play a critical role in microbiota-gut-brain communication. We have previously demonstrated that the depletion of regulatory T cells (Tregs), a major

immune suppressive cell, attenuates the behavioural effects of JB-1 in mice. Furthermore, the adoptive transfer of JB-1 induced Tregs mimics the anxiolytic effects of the bacteria (Y. Liu, Mian, Neufeld, & Forsythe, 2020), suggesting that gut microbes can affect brain function through promotion of Treg activity. Within the brain, microglia are the major immune cell. Microglia are activated following brain injury, infection, and inflammation and functions of the cell include clearance of cellular debris and dead neurons through the process of phagocytosis and promotion of inflammatory responses. In the healthy brain, microglia are also robustly dynamic and survey the brain parenchyma (Wake, Moorhouse, & Nabekura, 2011). Recent studies have identified that the density and morphology of microglia are altered, and function primed, following chronic psychological stress (Niraula, Sheridan, & Godbout, 2017; Tynan et al., 2010) and there is evidence of a causal relationship between microglial activation and anxiety-like behaviour.

The vagus nerve has been demonstrated to modulate both microglial activation and the induction of Tregs in the periphery however, to our knowledge these relationships have not been studied from the perspective of gut-brain communication and its effects on behavior. Here we assess how sub-diaphragmatic vagotomy influences JB-1 effects on HPA axis responsiveness, the peripheral immune profile and activation of hippocampal microglial, in relation to the anxiolytic effects of the bacteria.

5.2. Methods

5.2.1. Animals. Male BALB/c mice, 6-8 weeks old were acquired (Charles River, Montreal, QC, Canada) and allowed to acclimatise to the housing facility for at least 1 week before surgery. The BALB/c strain was chosen as they are considered as having high trait stress and previous studies have demonstrated baseline behaviors to be sensitive to *Lactobacillus rhamnosus* JB-1 induced modulation (Bravo et al., 2011; Y. Liu et al., 2020; McVey Neufeld et al., 2018). Mice were housed 3 per cage, at 25 ℃ on a 12h light/dark cycle (7am to 7pm), with access to regular chow and water ad libitum. All experimental procedures were carried out in accordance with Canadian Council on Animal Care guidelines and were approved by the McMaster Animal Research Ethics Board.

5.2.2. Vagotomy. Sub-diaphragmatic vagotomy was conducted as previously described (Bravo et al., 2011; Neufeld et al., 2019). Briefly, mice were anesthetized with inhale isoflurane and placed in a supine position. A midline incision was made on the upper abdomen (approx. 2cm) was made to expose the stomach and lower esophagus. Both vagus nerve branches in front of and behind the esophagus, along with the surrounding connective tissue were dissected. In order to reduce the gastric retention in vagotomised animals, pyloroplasty was performed with a 0.5 cm incision made across the pylori and then sutured tightly. For mice in the sham groups, only laparotomy and pyloroplasty were performed. All surgical procedures were conducted under a microscope by a single researcher. Animals

were postoperatively monitored and allowed for recover for 21 days prior to further treatment.

5.2.3. Probiotic Administration. *Lactobacillus rhamnosus* JB-1 was a gift (Alimentary Health Ltd., Cork, Ireland) and administered as previously described (Y. Liu et al., 2020). Briefly, the frozen powder of the bacteria was dissolved in normal drinking water in a 50 ml conical tube with fitted rubber sipper at $1x10^9$ CFU per day based on the previous studies determining each mouse consumes on average 3 ml per day (McVey Neufeld et al., 2018). Drinking water alone was provided in the same tubes to the control groups. The bacteria solution was renewed daily and administered for 28 days until the end of behavioral testing.

5.2.4. Behavioral Testing. The behavioral tests were initiated after 14 days of probiotic ingestion, and a 3-day rest period was provided between tests to reduce the interference of the previous test on the following one. Mice were brought to the testing room and allowed 1 hour to habituate to the environment. The testing apparatus was thoroughly cleaned with distilled water and dried before each test. All behavior tests were conducted between 9am and 11am.

Tail Suspension Test. Mice were suspended up-side-down by the tail using tape (2cm for taping and 15 cm for hanging) to ensure they were unable to escape or touch any surface. Following suspension activity was video recorded for 6 minutes and analysed by a researcher blinded to the treatment groups, the duration of

freezing behavior was assessed and calculated as percent of total suspension time, as previously described (McVey Neufeld et al., 2018).

Open Field Test. A transparent Plexiglas enclosure with side length of 18x38cm was used as the open field arena. A single mouse was brought into the enclosure and allowed 30 minutes of free exploration. General locomotor activity, as wells as the time, distance and entries to the center, were recorded by Motor Monitor Software (Kinder Scientific, Poway, CA, USA) as previously described (Bravo et al., 2011).

Light-Dark Test. The light-dark test utilized the same enclosure as the open field test. The addition of a black Plexiglas box (side length: 18x19 cm) on one side of the field was used to provide a dark zone. Mice were put in the dark zone at first and were allowed to cross two zones through an open doorway. Free exploration was allowed for 10 minutes and time spent, distance travelled and entries to light and dark zones was recorded by Motor Monitor Software (Kinder Scientific, Poway, CA, USA) (Bharwani et al., 2017).

Elevated Plus Maze. A black Plexiglas plus sign apparatus, with two open arms (30x5 cm), two closed arms (30x5x15 cm), and a central platform (5x5 cm), elevated 60 cm above floor, was used in this experiment. Mice were placed on the central platform with nose facing an open arm and allowed 5 minutes of free exploration. Time spent, distance travelled and entries into open and closed arms were recorded by Motor Monitor Software (Kinder Scientific, Poway, CA, USA) (Bravo et al., 2011).

5.2.5. Acute Restraint Stress and Serial Blood Collection. Five days after elevated plus maze, animals were brought to a testing room and allowed 30-minutes habituation. Mice were then placed on a table and their tail tip was cut with a scalpel blade and about 20 µl blood was collected for baseline corticosterone test (T0), via capillary tube. The tail blood was then decanted into a 1.5 ml collection tube containing 5 µl ethylenediaminetetraacetic acid (EDTA). Animals were then placed in wire mesh restrainers and singly house for 30 minutes. After restraint, mice were released, and blood collected as described above (T30). The mouse was then singly housed in a cage for another 30 minutes before a third blood collection (T60). All restraint stress blood collection procedures occurred between 9am and 12pm, in order to avoid circadian variation of corticosterone. Blood was then centrifuged in 10,000 rpm for 10 minutes and plasma was carefully collected and stored in a -80°C freezer until ELISA assay (Enzo Life Sciences, Toronto, ON, Canada) for corticosterone, following directions of the manufacturer (McVey Neufeld et al., 2018).

5.2.6. Assessment of Iba-1$^+$ Microglia. Following restraint stress and blood collection, mice were anaesthetized and perfused with phosphate buffered saline solution (PBS) through intracardiac perfusion. The brain was then removed and immersed in 4% paraformaldehyde (PFA) overnight, before being transferred to 30% sucrose in PBS until the brain sank. The brains were snap frozen with

isopentane and stored in a -80°C freezer for storage. The brains were then sectioned by cryostat in -20°C with a thickness of 25 μm. Then the sections were washed by floating in PBS and then blocked in 0.2% Triton-X 100/0.01 M PBS (PBST) containing 5% bovine serum albumin for 1 hour at 4°C. Sections were then incubated with primary antibody for ionized calcium-binding adaptor molecule 1 (Iba-1, 1:1000, rabbit anti-mouse, Abcam, Cambridge, MA, USA) at 4°C, on a shaking bed overnight. Sections were then washed 3 times in PBST and incubated with AlexaFluor 594-conjugated goat anti-rabbit secondary antibody (1:400, Abcam, Cambridge, MA, USA) for 1 hour at room temperature. Following another 3x wash with PBST sections were mounted on gelatin coated slides and the nucleus of cells were stained by DAPI (Vector Laboratories, Burlingame, CA, USA). Hippocampal sections were imaged under Zeiss AxioImager Z1 microscope (AxioCam, Köln, Germany) and analysed using ImageJ software. Ten sections per hippocampus per animal was analyzed, and the population Iba-1^{+} microglia was calculated by relative fluorescence intensity unit (RFU) in manually designed regions of interest and computing an average RFU per section (RFUs include CA1, CA3 and DG), for RFU is previously used to indicate the density of microglia in hippocampus (Iulita et al., 2018; Sui et al., 2016). Values of the intensity were compared to Sham/Water group after background subtraction.

5.2.7. Serum Cytokine/Chemokine Analysis. Trunk blood was collected directly after euthanization from common carotid artery, and serum was made after

centrifuge. The levels of interleukin (IL) -1α, -1β, -2, -4, -5, -6, -10, -12, -13, -17A, chemokine (C-X-C motif) ligand-1, -2, -5, C-C motif ligand-2 (CCL-2), and tumor necrosis factor-alpha (TNF-α) were determined through Multiplex Cytokine/Chemokine Analysis (Eve Technologies, Calgary, Canada.

5.2.8. Flow Cytometry. Following euthanization, the spleen was collected in 5% BSA-RPMI media 1640, a single cell suspension of splenocytes was made and red blood cells was lysed by RBC lysis buffer. Splenocytes were stained for $Ly6C^{hi}$ monocytes (CD11b-PE, Ly6C-APC), activated dendritic cells (CD11c-APCeFluor780, CD80-PerCP, CD86-APC, MHC II-FITC), and regulatory T cells (CD3-APC-eFluor780, CD4-FITC, CD25-APC, Foxp3-PE), with $1x10^6$ cells for each stain. All conjugated antibodies were obtained from Invitrogen, ThermoFisher Scientific, USA, and diluted by 1:200 with PBS. The flowcytometry results were analysed by FlowJo 10.0 software (FlowJo LLC, USA), with doublets and cell debris excluded by forward scatter (FSC) and side scatter (SSC) gating.

5.2.9. Statistical Analysis. All data were analysed by GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) by two-way ANOVA with Tukey's post hoc tests. Results are illustrated as mean ± standard error of the mean (SEM) in all figures. F value of factor interaction and Hedge's g value were calculated. p value smaller than 0.05 was considered as statistically significant.

5.3. Results

5.3.1. Vagotomy reduced the anxiolytic effects by treatment of *Lactobacillus rhamnosus* JB-1.

The overall timeline of the experiment is illustrated as Figure 1. Tail suspension test (TST) was conducted to evaluate the learned helplessness as an indicator or depressive behavior. Neither vagotomy or JB-1 lead to significant difference among groups on the percentage of immobile time in TST ($F(1, 43)=0.7580$, Sham/Water: 18.9±3.9, Sham/JB-1: 21.0±3.0, $g=0.1717$, $p=0.9791$; Vx/Water: 27.6±4.9, $g=0.5835$, $p=0.3830$, compared to Sham/Water; Vx/JB-1: 23.1±3.2, $g=0.3241$, $p=0.8385$, compared to Vx/Water, n=11-12, Figure 2A).

We then analysed the anxiety-like behavior using the open field test (OFT), light-dark test (LDT) and elevated plus maze (EPM). In the OFT, there was no significant difference either in the basic movements or activities in the center area (Figure S1A-D). In the LDT, there was a significant increase of light zone activity with JB-1 treatment in sham surgery mice (time spent in light zone: $F(1, 44)=10.2$, Sham/Water: 84.8±16.3s, Sham/JB-1: 183.5±24.2s, $g=1.3779$, $p=0.0048$; distance moved in light zone: $F(1, 44)=9.821$, Sham/Water: 229.6±46.2, Sham/JB-1: 457.9±50.0, $g=1.3700$ $p=0.0056$; entries into light zone: $F(1, 44)=7.009$, Sham/Water: 13.8±1.7, Sham/JB-1: 24.9±2.9, $g=1.3280$, $p=0.0049$, n=12, Figure 2B-D), these effects of JB-1 were not observed in vagotomized mice (time spent in light zone: Vx/Water: 134.0±15.1s, Vx/JB-1: 105.1±21.2s, $g=0.4536$, $p=0.7241$; distance moved in light zone: Vx/Water: 343.9±34.5, Vx/JB-1: 284.0±51.375,

g=0.3952, p=0.7936; entries into light zone: entries into light zone: Vx/Water: 18.9±2.1, Vx/JB-1: 18.3±1.8, g=0.0858, p=0.9976). In the EPM, we found significant increase of time spent and distance moved in open arms after JB-1 ingestion in sham operated mice (time spent in open arms: F(1, 43)=3.617, Sham/Water: 8.2s±2.7s, Sham/JB-1: 17.8±2.7s, g=1.0509, p=0.0275; distance moved in open arms: F(1, 43)=2.866, Sham/Water: 47.9±10.8, Sham/JB-1: 115.4±19.0, g=1.2594, p=0.0140; Figure 2E&F), However JB-1 treatment in vagotomised mice did not alter behavior in the EPM (time spent in open arms: Vx/Water: 6.7±1.7s, Vx/JB-1: 7.6±2.0s, g=0.1323, p=0.9934; distance moved in open arms: Vx/Water: 55.9±11.1, Vx/JB-1: 73.2±16.2, g=0.3588, p=0.8395; also see Figure S1E for entries of open arms).

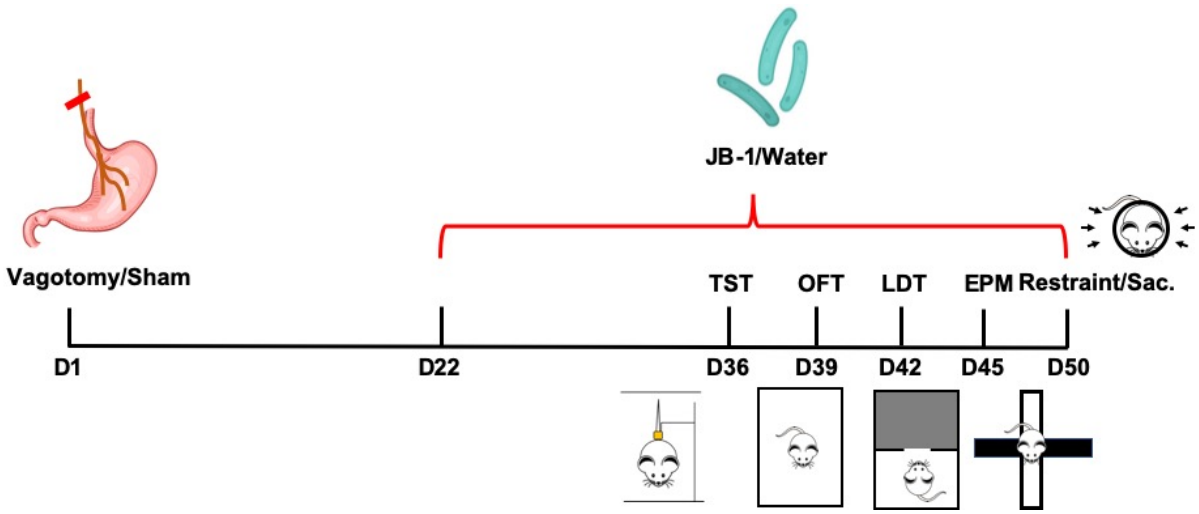


Figure 1. Timeline of the experiment. All mice were under vagotomy and sham surgery at D1. *Lactobacillus rhamnosus* JB-1 and water were given continuously until D50 since D22. A series of behavior tests were conducted from D36 to D45.

The mice were stressed by 30-minute acute restraint stress at D50, then euthanized for tissue collection.

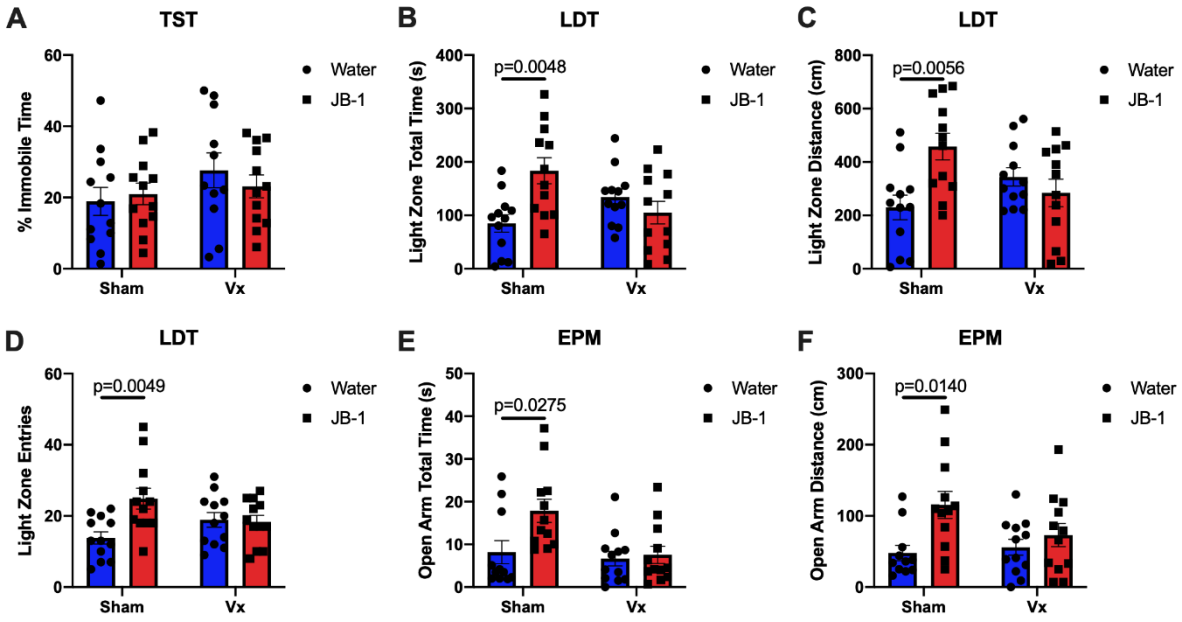


Figure 2. Vagotomy reduced the JB-1 induced anxiolytic effects. (A) Percentage of immobile time spent in TST (n=11 in Vx/Water, n=12 in the other groups). **(B)** Time spent in light zone in LDT (n=12). **(C)** Distance traveled in light zone in LDT (n=12). **(D)** Entries into light zone in LDT (n=12). **(E)** Time spent in open arms in EPM (n=11 in Sham/Water group, n=12 in the other groups). **(F)** Distance traveled in open arms in EPM (n=11 in Sham/Water group, n=12 in the other groups).

5.3.2. The ability of *Lactobacillus rhamnosus* JB-1 to attenuate stress induced blood corticosterone level was prevented by vagotomy.

There was no significant difference in CORT levels between the groups at baseline (T0) or immediately following restraint stress (T30). However, 30 minutes following stress there was a significant reduction in CORT levels in the Sham/JB-1 group, compared to Sham/Water group ($F(1, 28)=0.9869$, Sham/Water: 21534.969±1649.209 pg/ml, Sham/JB-1: 12437.658±1694.507 pg/ml, $g=1.9237$, $p=0.0054$). However, JB-1 failed to induce the decrease of corticosterone level in vagotomized mice, compared to Vx/Water group (Vx/Water: 23776.346±1389.674 pg/ml, Vx/JB-1: 18174.040±2203.273 pg/ml, $g=1.0753$, $p=0.1341$, Figure 3).

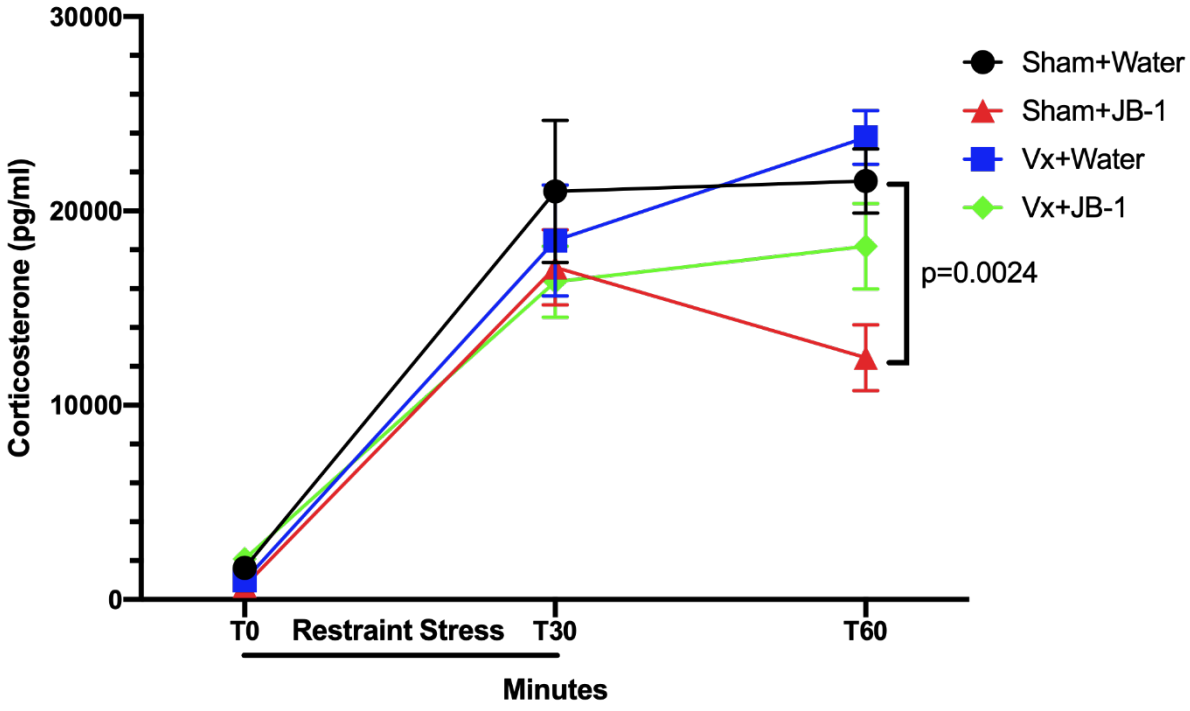


Figure 3. JB-1 reduced plasma corticosterone level 30 minutes after acute restraint stress, but did not show decreasing effect in vagotomized mice (n=8).

5.3.3. Peripheral immune cells were affected by vagotomy and JB-1.

Splenocytes were collected and processed for flowcytometry tests in order to analyse peripheral immune changes. The general $CD4^+$ T cells population showed no significant difference among groups (Figure S2A). JB-1 feeding led to a significant increase in $CD4^+CD25^+Foxp3^+$ T cells (percentage of $CD4^+$ T cells, $F(1, 19)=1.523$, Sham/Water: 19.9±0.8%, Vx/Water: 24.3±0.8%, $g=2.2269$, $p=0.0134$), however, this increase was not seen in vagotomized mice (Vx/Water: 19.3±1.3%, Vx/JB-1: 21.5±0.6%, $g=0.8587$, $p=0.4171$, Figure 4A).

Next, we found that there was no significant difference in $CD11b^+$ cells among 4 groups with surgery and feeding (Figure S2B). The level of $CD11b^+Ly6C^{hi}$ monocytes were increased after vagotomy, with or without JB-1 administration (percentage in $CD11b^+$ cells, $F(1, 20)=0.2013$, Sham/Water vs. Vx/Water: 8.6±0.7% vs. 13.5±1.0%, $g=2.2645$, $p=0.0037$; Sham/JB-1 vs. Vx/JB-1: 8.2±0.5% vs. 12.2±1.1%, $g=1.9691$, $p=0.0154$), Figure 4B). CCR2 expression did not alter significantly after vagotomy and JB-1 treatment (Figure S2C).

Furthermore, we also tested the population of dendritic cells as an index of antigen presenting process. The level of general $CD11c^+MHC\text{-}II^+$ cell population showed no significant difference among four groups (Figure S2D), and difference

was not found in CD80 expression (Figure S2E), but $CD86^+$ dendritic cells reduced significantly after vagotomy, with or without JB-1 (percentage in $CD11c^+MHC\text{-}II^+$ cells, F(1, 20)=0.8545, Sham/Water vs. Vx/Water: 9.0±1.0% vs. 6.5±0.3%, g=1.3131, p=0.0401; Sham/JB-1 vs. Vx/JB-1: 9.1±0.4%, vs. 5.5±0.3%, g=4.0129, p=0.0022; Figure 4C). No significant difference was found in serum cytokine/chemokine levels between the treatment groups (See Table S1).

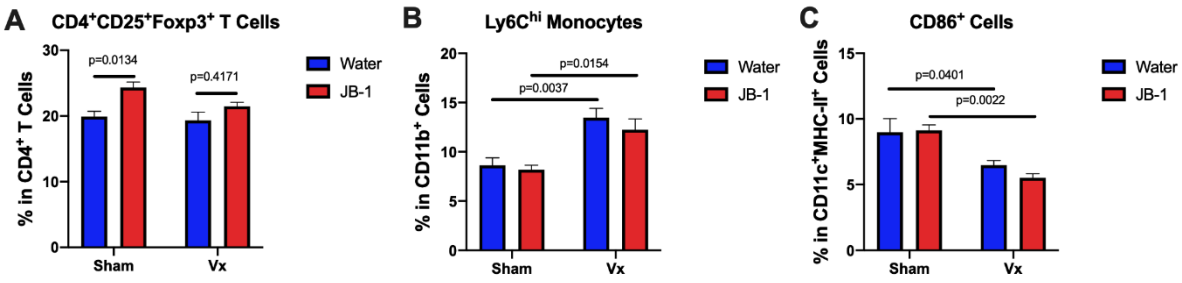
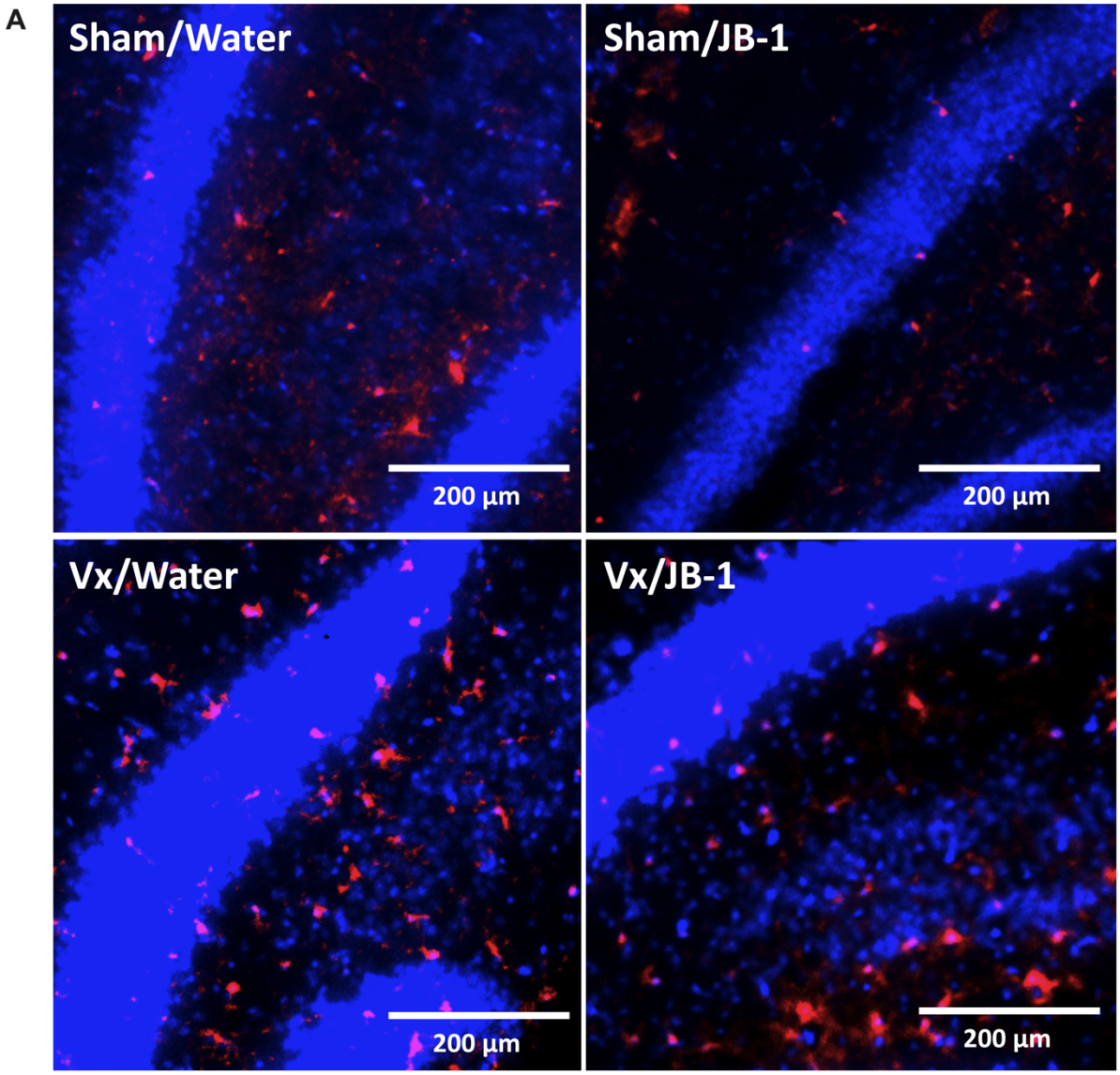


Figure 4. Vagotomy and JB-1 administration altered peripheral immune cell levels. (A) Percentage of $Foxp3^+$ regulatory T cells in $CD4^+$ T cells (n=5 in Vx/JB-1, n=6 in the other groups). **(B)** Percentage of $Ly6C^{hi}$ monocytes in $CD11b^+$ cells (n=6). **(C)** Percentage of $CD86^+$ cells in dendritic cells (n=6).

5.3.4. Hippocampal Iba-1^+ microglia were decreased by *Lactobacillus rhamnosus* JB-1 and increased by vagotomy.

The RFU intensity of Iba-1+ was significantly decreased after JB-1 treatment in the sham surgery group (F(1, 20)=1.630, Sham/Water: 1.000±0.046, Sham/JB-1: 0.795±0.0036, g=2.0363, p=0.0351). However, vagotomy led to

significant increase of Iba-1 intensity in the hippocampus (Vx/Water: 1.242±0.059, g=1.8707, p=0.0098; Vx/JB-1: 1.157±0.044, g=3.6519, p=0.0002), an effect that was not significantly altered by JB-1 treatment (g=0.6645, p=0.7669, Figure 5B).



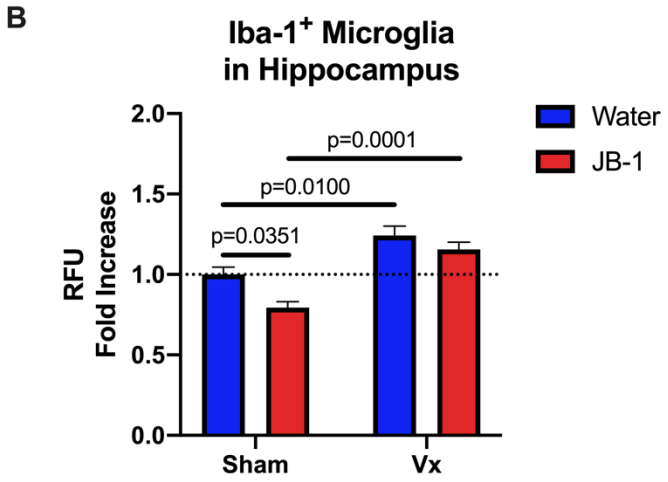


Figure 5. JB-1 led to reduction, but vagotomy led to an increase in Iba-1$^+$ microglia in the hippocampus. (A) Hippocampal sections stained for Iba-1 (red) and DAPI (blue). **(B)** Relative fluorescence intensity units (RFU) of Iba-1 in the hippocampus, illustrated as fold increase compared to Sham/Water group (n=5-6).

5.4. Discussion

It has previously been demonstrated that the anxiolytic and antidepressant-like effects of *L. rhamnosus* JB-1 are dependent on an intact vagus nerve connection between gut and brain (Bravo et al., 2011). Here we demonstrate, for the first time, loss of vagal integrity also inhibits JB-1 induced modulation of the HPA axis response and the induction of regulatory T cells, the latter of which is critical to the effects of the bacteria on behaviour (Liu et al., 2020). In addition, subdiaphragmatic vagotomy alone causes an increase in activated splenic monocytes and hippocampal microglia. The increase in activated microglia in the

hippocampus following vagotomy counters the decrease in these cells associated with the effects of *L. rhamnosus* JB-1 on behaviour.

The vagus nerve responds to changes in gut luminal content and signals these changes to the brain. Chemoreceptors expressed by vagal neurons can be activated directly by substances such as short chain fatty acids that can be transported across the epithelial barrier or by paracrine mediators such as 5-HT, histamine, CCK, or glucagon-like peptides released by the various mucosal epithelial layer taste cells (Forsythe, Kunze, Bienenstock, 2014). In addition, enteric neurons can signal sensory information to the vagus nerve via an intramural synapse between the intrinsic primary afferent neurons (IPAN) and vagal afferents. Indeed, this IPAN to vagus sensory synapse has been identified as playing a critical role in the rapid activation of vagal afferent fibers following introduction of JB-1 to the gut lumen (Perez-Burgos et al., 2013) that in turn leads to activation of neurons in widespread brain regions (Bharwani et al., 2020).

In light of the role for vagal afferents in transmitting sensory information from the gut to the brain, observations that vagotomy prevents the effect of JB-1 (Bravo et al.) and other microbes (Malick et al., 2015; Bercik et al., 2011) on behavior are generally interpreted as evidence that the vagus nerve acts as a “direct line” between the intestinal bacteria and neural circuitry influencing anxiety and depression.

However, it is clear that the immune system also plays a critical role in mediating the antidepressant and anxiolytic-like effects of JB-1. We recently

demonstrated that cells within the $CD4^+CD25^+$ population of JB-1 treated mice, most likely T regulatory cells (Tregs), are necessary and sufficient to mediate behavioural effects of the microbe.

In our current study, we found that the JB-1 induced increase the Treg population, as defined by $CD4^+CD25^+$ cells expressing Foxp3, while evident in the sham operated mice was prevented by vagotomy.

The immunomodulatory role of the vagus nerve is well established, with the cholinergic anti-inflammatory reflex the best described mechanism for vagal regulation of immune cell function. In this reflex, afferent vagal fibers sense inflammatory signals from the periphery, relaying information for central integration in the NTS and dorsal motor nucleus with subsequent activation of efferent vagus nerve fibers and suppression of cytokine production via the release of the parasympathetic neurotransmitter acetylcholine (ACh) (Goehler et al., 2000). Regulatory T cell development and function has been demonstrated to be under vagal control, with vagal nerve stimulation resulting in increased numbers (Teratani et al., 2020) and activation of nicotinic ACh receptors leading to enhanced regulatory function (Wang et al., 2010) of the cells.

Given the critical role for Treg in mediating the behavioral effects of JB-1, the results of the current study suggest that the absence of anxiolytic effects following vagotomy may be due to blockade of a vagus nerve dependent immunoregulatory reflex stimulated by the bacteria. Such a vagus nerve dependant induction of $CD4^+CD25^+Foxp3^+$ T cells has been demonstrated to underlie

enhanced wound healing in mouse following oral treatment with a *L. reuteri* strain (Poutahidis et al., 2013).

Our study also identifies constitutive effects of the vagus nerve on the immune system, the absence of which may interfere with the ability of gut microbes to modulate brain function. The population of activated peripheral monocytes was increased following vagotomy, an effect that was not modulated by JB-1. Vagal regulation of monocytes/macrophages is well described (Anderson, Tracey, 2012) and selective nicotinic receptor agonists to attenuate cytokine production of human monocytes (Rosas-Ballina et al., 2009). Activated monocytes also act as a link between the peripheral immune system and CNS. In mice, repeated social defeat induces an increase in circulating activated monocytes, and recruitment of these monocytes to the brain serves to amplify neuro-inflammation in response to stress and promotes anxiety-like behaviour (Wohleb et al., 2013). A high level of circulating activated monocytes has also been reported as an indicator of stress susceptibility in mice (Gururajan et al., 2019).

Microglia are resident macrophages in the CNS and communicate with neurons and with cells of the immune system. Studies have provided evidence of the involvement of microglia in psychiatric disorders. Repeated social defeat in mice, a model for post-traumatic stress disorder (PTSD), led to an increase in deramified microglia in the medial amygdala, prefrontal cortex and hippocampus, correlated with enhanced ability of the cells to produce IL-6, TNF-α, and chemokine (C-C motif) ligand-2 (CCL-2) (Wohleb et al., 2011). Microglial depletion was found

to be able to alleviate anxiety and PTSD-like symptoms in the mice symptoms as illustrated by increased open arm entries in EPM and decreased freezing time in contextual fear tests (Li et al., 2021). Our current study found that the probiotic *L. rhamnosus* JB-1 could reduce the relative intensity of Iba-1, a marker of activated microglia, in the hippocampus in the sham surgery group. This decrease in activated microglia was correlated with reduced anxiety-like behaviors observed in the LDT and EPM. Consistent with our findings, increased microglial activation in the hippocampus of mice following chronic unpredictable mild stress (L.-L. Liu, Li, Su, Wang, & Jiang, 2019), was, negatively associated to the emergence of anxiety- and depressive-like behaviors (Berry et al., 2012) and there is good evidence of a causal relationship between microglial activation and anxiety-like behaviour (Wohleb et al., 2011). Sub-diaphragmatic vagotomy alone resulted in a marked increase of Iba-1 intensity in the hippocampus that was not attenuated by JB-1. These results support previous findings of Gallaher et al. who demonstrated that vagotomy results in enhanced microglial activation (as indicated by Iba-1^+ cells) in 'vagal structures', including the nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus nerve (DMV), and nodose ganglia (NG); all regions with connections to the hippocampus, one of the central structures in emotional control (Gallaher, et al., 2012). Thus, the anxiolytic effects of JB-1 may be mediated by a down-regulation of microglial activity, an effect that is masked following vagotomy due to loss of a constitutive vagal inhibition of microglial activation. Our study provides novel evidence to support the concept that the role of the vagus nerve in

mediating the anxiolytic effects of the psychoactive bacteria, *Lactobacillus rhamnosus* JB-1, is not only as a "direct line" from gut to brain. Disruption of vagal integrity leads to loss of the ability of JB-1 to induce $CD4^+CD25^+Foxp3^+$ regulatory T cells, an essential component of the behaviour modifying effects of the bacteria (Liu, Mian, Neufeld, & Forsythe, 2020). These results suggest that JB-1 induction of Tregs may be the result of activation of a vagus nerve dependant immunoregulatory reflex. Of note, the SSRI antidepressant fluoxetine has been demonstrated to activate a vagal anti-inflammatory pathway (Ondicova et al., 2019) and vagotomy inhibits the ability of oral fluoxetine to decrease learned helplessness behavior of mice in the TST (Neufeld et al., 2019), although the relationship between these two observations has not been explored.

The anxiolytic effects of JB-1 is associated with a decrease in microglial activation in the hippocampus and while the suppressive effect of Treg on microglia has been demonstrated (Xie et al., 2015), future studies should investigate the functional relationship between enhanced peripheral Treg and activated hippocampal microglia in relation to microbe-gut-brain signaling. Similarly, a potential causal relationship between the increase in activated microglia and peripheral monocytes following vagotomy should be explored. Based on the current study, it is clear that in order to determine the true role of the vagus nerve in communication between gut microbes and the brain will require a dissection of afferent and efferent signaling pathways and their relative contribution to neuroimmune crosstalk in the CNS.

Declaration of Competing Interest

The Authors declare no competing interests.

Contributors

Y.L. and P.F., designed the experiments. Y.L., D.S. and K.-A.M.N. performed experiments. Y.L., D.S., M.F.M. and K.-A.M.N. analysed the data and constructed the figures. Y.L. and P.F. wrote and edited the manuscript, which was reviewed before submission by all authors.

Acknowledgements

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5.5. Supplementary Information

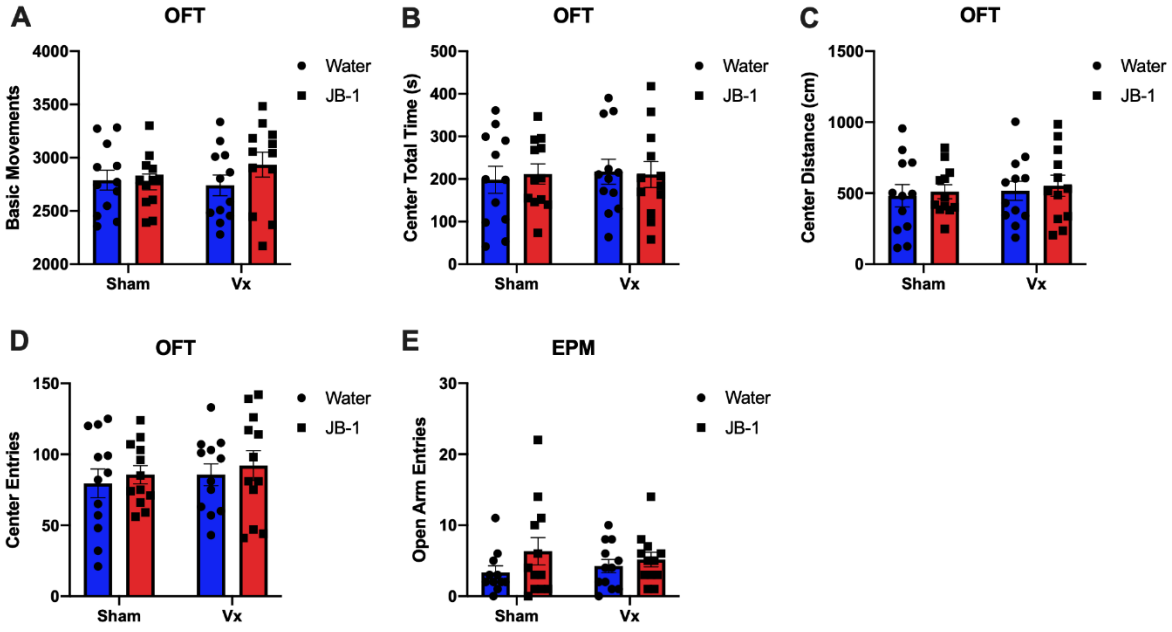

Figure S1. Supplementary behavior data. (A) Basic movements in OFT (n=12). **(B)** Time spent in center area in OFT (n=12). **(C)** Distance traveled in center are in OFT (n=12). **(D)** Entries into center area in OFT (n=12). **(E)** Entries into open arms in EPM (n=11 in Sham/Water, n=12 in the other groups).

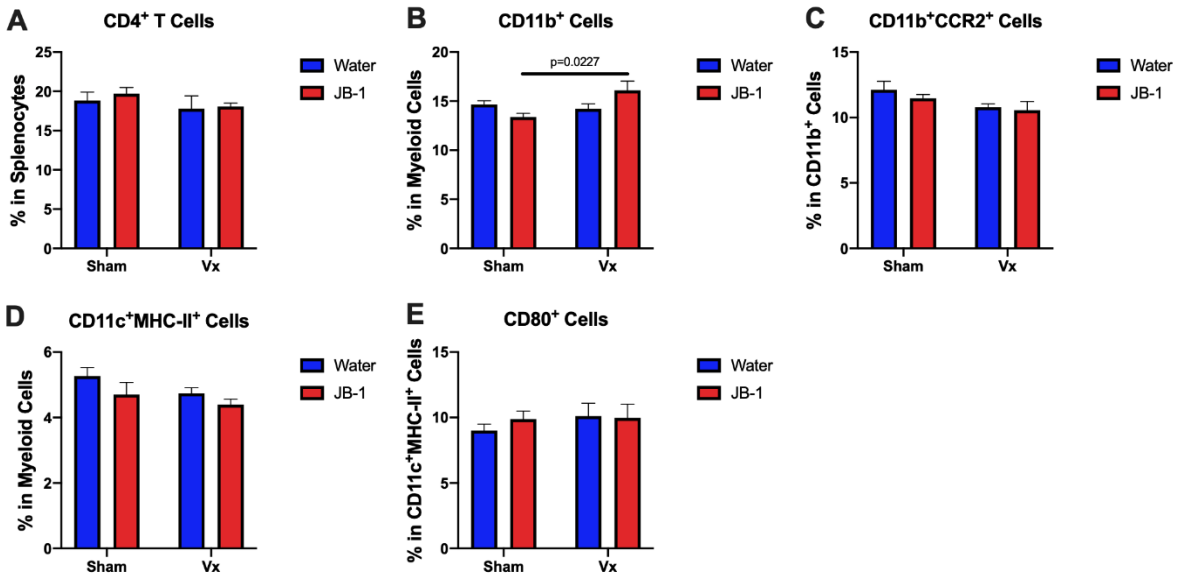

Figure S2. Supplementary flow cytometry results of splenocytes (n=6). **(A)** Percentage of $CD4^+$ T cells in lymphocytes. **(B)** Percentage of $CD11b^+$ cells in myeloid cells. **(C)** Percentage of $CD11b^+CCR2^+$ cells in $CD11b^+$ cells. **(D)** Percentage of $CD11b^+MHC\text{-}II^+$ cells (dendritic cells) in myeloid cells. **(E)** Percentage of $CD80^+$ cells in dendritic cells.

	Sham/Water	**Sham/JB-1**	**Vx/Water**	**Vx/JB-1**
IL-1α	24.833±8.048	27.172±5.039	24.331±9.065	20.447±4.722
IL-1β	0.385±0.081	0.293±0.044	0.258±0.060	0.313±0.063
IL-2	2.974±0.344	3.346±0.398	3.756±0.868	4.049±1.023
IL-4	0.093±0.030	0.082±0.025	0.249±0.148	0.116±0.033
IL-5	9.225±1.610	7.133±1.246	7.242±1.493	7.856±1.281
IL-6	18.054±4.180	21.033±5.256	15.536±3.800	21.551±3.759
IL-7	26.256±14.349	14.410±8.733	10.511±7.429	3.179±0.909
IL-10	6.778±2.367	5.542±1.302	7.836±3.595	3.960±0.767
IL-12	26.758±19.449	5.902±3.120	21.655±10.327	5.514±2.492
IL-13	6.668±0.641	6.937±0.851	5.349±0.756	4.870±1.349
IL-17A	3.368±0.427	3.401±0.639	3.769±0.657	2.312±0.499
CXCL-1	68.522±19.558	99.242±27.031	64.216±20.617	89.348±22.984
CXCL-2	47.288±4.441	41.711±5.149	34.490±5.323	40.126±10.318
CXCL-5	979.736 ±294.596	1466.704 ±308.794	1122.402 ±415.096	1026.632 ±283.538
CCL-2	4.737±0.967	5.253±0.988	2.824±0.539	3.838±0.762
TNF-α	2.513±0.151	3.248±0.267	2.433±0.360	3.139±0.344

Table S1. Cytokines/chemokines results of serum by Multiplex Analysis. Data were presented as pg/ml (n=7-12).

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CHAPTER 6. DISCUSSION

6.1. Summary of findings

This thesis investigates phenomenology and mechanisms related to the effects of *Lactobacillus rhamnosus* JB-1 on brain function and behaviour. Two major regulatory components in microbiota-gut-brain axis, $CD4^+CD25^+$ regulatory T cells and vagus nerve, are studied, as is the effect of early post-stress administration of JB-1 in mice exposed to chronic social defeat. In Chapter 3, C57BL/6 exposed to 10-day chronic social defeat were orally administered with JB-1 or sertraline for 4 weeks. Contrary to previous findings that consumption of JB-1 before and during the trauma-like event helped to prevent fear and anxiety-like behaviors, early-stage, post-stress treatment increased persistence of both aggressor avoidance and reduced sociability. These behavioural effects were correlated with altered gene expressions in the hippocampus, including CRHR-1, GR, BDNF, and $GABA_{A2}$. We also observed reduced levels of $Ly6C^{hi}$ monocytes and serum CCL-2 in the JB-1 fed group. These results demonstrated that, under certain circumstances, probiotic could perpetuate, rather than prevent behavioral deficits following stress exposure. In Chapter 4, we used anti-CD25 antibody to deplete $CD4^+CD25^+$ regulatory T cells (Tregs) in *Lactobacillus rhamnosus* JB-1 fed BALB/c mice, a strain of mice considered usually with high trait anxiety, and found that the anxiolytic and anti-depressive effects induced by JB-1 were attenuated. Furthermore, the JB-1 induced decrease in $Ly6C^{hi}$ monocytes, an inflammatory

population of immune cells commonly proliferated by stress, was not observed in the Treg depleted group. Adoptive transfer of JB-1 induced Tregs from donor mice into JB-1 naïve mice, led to similar behavioral effects as JB-1 feeding. These results indicate that Tregs are necessary and sufficient for behavioral effects of gut microbes, possibly by inhibition of excessive activation of inflammatory monocytes. In Chapter 5, we applied long-term administration of JB-1 in vagotomised mice, and demonstrated JB-1 induced promotion of peripheral Tregs, reduction in microglia activation in the hippocampus, and attenuation of HPA axis reactivity, were inhibited after vagotomy as were the anxiolytic effects of the bacteria. These results indicate that vagus nerve integrity is required for the immune changes associated with, and causally related to, the effects of *L. rhamnosus* HPA axis activity and behavior.

6.2. Prevention or exacerbation: a proper time window to modulate stress responses by probiotics.

About 70-80% people will be exposed to severe trauma-like event, at least once in their lifetime, and about 20% of this population will suffer from PTSD or PTSD-like symptoms (de Vries & Olff, 2009; Knipscheer et al., 2020). The current diagnosis of PTSD requires at least one-month period of clinically confirmed symptoms (including flashback and social deficits, etc.) following trauma exposure. The purposes of aftermath intervention for people exposed to trauma-like event are 'to preserve life, prevent further harm, and promote recovery' (Nash & Watson,

2012). An evidence-based review of early intervention and prevention of PTSD, by Birur et al., examined 21 clinical studies using pharmaceutical treatment. Contrasting results were found among studies. For example, administration of propranolol within 6 hours showed more preventive effects than those who started the treatment several days after the exposure to trauma (Birur, Moore, & Davis, 2017). While early-stage administration of the antidepressant escitalopram, a member of the SSRI family, did not result in positive clinical outcomes. One study demonstrated that among the trauma-exposed people, both escitalopram and placebo showed significant reduction in PTSD symptoms, with no significant difference among groups (Suliman et al., 2015). Shalev et al. also found escitalopram did not have more beneficial effects than placebo (Shalev et al., 2012). Benzodiazepines, as well-known anxiolytic and sedative drugs, given within 3 weeks of trauma, did not generate difference compared to placebo (Gelpin, Bonne, Peri, Brandes, & Shalev, 1996). Temazepam treatment has even been demonstrated to lead to higher diagnostic rate of PTSD than placebo group (Mellman, Bustamante, & Fins, 2002). Birur et al., concluded that even though several pharmaceutical agents have been used in PTSD, so far only hydrocortisone administered prior to trauma-like events has demonstrated effectiveness in the reduction of stress symptoms and the onset of PTSD (Birur et al., 2017).

In this thesis, we demonstrated that 4-week administration of JB-1 within 48 hours after 10-day chronic social defeat did not lead to behavioral benefits in

C57BL/6 mice. Instead, behavior was normalized in the control group, which showed increased social interaction with conspecific and aggressor mice. This indicated a natural recovery from exposure to trauma, that was prevented by both JB-1 or sertraline treatment. This raises the question why this early-stage window of probiotic or SSRI intervention has detrimental effects on PTSD associated symptoms in this model. Alboni et al. found that the effects of fluoxetine treatment on stressed mice depended on whether the environment was enriched or stressful, and they propose SSRIs do not really change the mood itself, but increased the brain plasticity allowing for alterations driven by the environment quality (Alboni et al., 2017). Administration of probiotic or SSRI, if followed by encounter of trauma-like event, which build a stressful environment, might make the brain quickly "adapt" to the new situation and enforce the abnormal behaviors including fear memory and social deficits. Next, we found that persistent fear memory of JB-1 or sertraline fed stressed animals was associated with reduced corticotropin-releasing hormone receptor-1 (CRHR-1) expression in the hippocampus. Extensive evidence indicates that the hippocampus is a major neural substrate underling contextual fear memory (Chaaya, Battle, & Johnson, 2018). Corticosterone synthesis during fear conditioning may be beneficial in stressed animals by facilitating extinction, while inhibition of corticosteroid synthesis during fear conditioning exacerbated fear extinction deficits (Keller, Schreiber, Stanfield, & Knox, 2015). Weber et al. found that decreased gene expression of CRHR-1 led to a phenotype characterized by fear sensitization and sustained fear (Weber et al., 2016). Cursano et al. found that

disruption of contextual memory consolidation ability, of mice exposed to thoracic trauma, was correlated with upregulated expression of CRHR-1, and a CRHR-1 antagonist led to prolonged memory (Cursano et al., 2020). Moreover, two types of corticosteroid receptors, mineralocorticoid and glucocorticoid receptors (MR and GR) were demonstrated to be related to fear memory. Using an antagonist of GR on contextual fear conditioned mice leading to prolonged fear extinction (Gourley, Kedves, Olausson, & Taylor, 2009). Ribeito et al. recently found that an MR agonist was able to facilitate the fear memory extinction (Ribeiro et al., 2020). Our study found a reduction in expression of both MR and GR in hippocampus, which might contribute to the impaired fear extinction in the group of stressed mice fed by JB-1 or sertraline. A Similar reduction was found in brain-derived neurotrophic factor (BDNF), which is among the key molecules involved in neuroplasticity changes related to learning and memory (Miranda, Morici, Zanoni, & Bekinschtein, 2019), and is demonstrated to enhance fear extinction. The HPA axis is also a target of BDNF (Andero & Ressler, 2012). Overall, JB-1 administration directly after social defeat could leads to reduced expression of several hippocampal molecules positively correlated to fear extinction, and led to persisted fear to stress. Consistent with mixed results of clinical trials focusing on early-stage pharmaceutical intervention, we could conclude that intervention by probiotic started within 48h after trauma is not able to reverse, but further impairs the fear extinction. Given the results presented in chapter 4 and 5 of this thesis, further studies should focus on immune changes following consumption of probiotic after

stress, and the functional link with molecular and functional alterations of brain. For example, does treatment alter Ly6C^{hi} monocytes in the periphery and activated microglia in the hippocampus in the trauma exposed mice, and whether the gene expression change of GR in hippocampus is consistent with changes in HPA axis reactivity, and how these changes mean to systemic immune alterations. Also, we should not exclude the existence of an effective early-stage window of intervention by probiotic, a shortened or prolonged gap between stress and treatment may provide effective protection against detrimental behavioural effects of chronic stress.

6.3. Immunoregulation is a protective mechanism against stress in the microbiota-gut-brain axis.

Immune balance is maintained by precise control of Inflammatory and anti-inflammatory activities.

Regulatory T cells (Tregs), are a subpopulation of CD4$^+$ T cells and marked by high expression of CD25 and fork-head box P3 (Foxp3), represent major immunoregulatory cells in humans and rodents. Many psychiatric disorders have been associated with exaggerated immune activation, and failure to control the inflammation through loss of Treg function may contribute to this process. Sommershof et al. found that PTSD patients displayed 48% reduction in the proportion of Tregs, which was correlated with enhanced proportions of CD3$^+$ central and effector memory T cells (Sommershof et al., 2009). On the other hand,

some pharmaceutical treatments that have immunoregulatory effects usually lead to better prognosis on psychiatric patients. For example, prostaglandin-endoperoxide synthase 2 (COX-2) inhibitor, or aspirin, was confirmed to have positive effects on patients with schizophrenia and major depression (Na et al., 2014; Sepehrmanesh et al., 2017). In animal research, maternal consumption of nonsteroidal anti-inflammatory drug could ameliorate placental inflammation and resulted offspring locomotor activity (Bronson & Bale, 2014). IL-10, a well-known anti-inflammatory cytokine, if injected intracerebroventrically on rats suffered from chronic force swim stress, could help to maintain normal saccharin preference, an index of depressive behavior, as well as travel distance/rearing in open field test, indexes of anxiety-like behaviors (Pan et al., 2013).

Ingestion of *Lactobacillus rhamnosus* JB-1 in BALB/c mice led to significant increase of Tregs (Karimi et al., 2012). Chronic ingestion of *Lactobacillus rhamnosus* JB-1 in mice before and during social defeat decreased stress-induced anxiety-like behavior and prevented deficits in social interaction, and these behavioral benefits were correlated with increased IL-10^{+} regulatory T cells in the spleen, suggesting strengthened immunoregulatory function by the probiotic may ameliorate behavioral deficits induced by stress (Bharwani et al., 2017). It was previously found that anti-CD25 monoclonal antibody led to anxiety-like and depressive symptoms in non-stressed C57BL/6 mice, suggesting Tregs is important in baseline modulation on behaviors (Kim et al., 2012). *Mycobacterium vaccae* ingestion prevented social deficits and anxiety-like behavior in mice with

chronic psychosocial stress, which were not seen in mice with depletion of Tregs (Reber et al., 2016). In this thesis, for the first time, we demonstrate that Tregs are necessary in the JB-1 induced anxiolytic and anti-depressive effects, as mice depleted with Tregs failed to show behavioral amelioration by JB-1. Moreover, supplementary treatment of Tregs from JB-1 treated donor mice could conduct similar effects as the probiotic itself (Y. Liu, Mian, Neufeld, & Forsythe, 2020). These findings addressed the important role which Treg played in the maintenance of homeostasis generated from probiotics, and emphasized that Tregs could be good target of therapy against stress related disorders.

Generally, although we know that Tregs have overall anti-inflammatory functions, we still need to recognize the exact downstream target of Treg to help us understand the whole map of immunoregulation in microbiota-gut-brain axis. Activated monocytes in blood and gastrointestinal tract associated lymph tissue have been identified to communicate between the immune system and the brain. It was observed that increased circulating $Ly6C^{hi}$ monocytes in mice following social defeat, and neuro-inflammation would be amplified in response to stress through the recruitment of the monocytes, finally promote anxiety-like behaviors (Wohleb et al., 2013). Furthermore, higher level of $Ly6C^{hi}$ monocytes could indicate higher susceptibility to stress in mice (Gururajan et al., 2019). Tregs could limit the inflammation induced by activated monocytes effectively. When co-cultured with Tregs, monocytes displayed typical immunosuppressive features, including up-regulated expression of CD206 (mannose receptor, an indicator of resolution of

inflammation), reduced expression of human leukocyte antigen DR (HLA-DR, a marker of effector T cell activation), and reduced capacity to respond to lipopolysaccharide (LPS, a commonly used stimulator of inflammation) (Tiemessen et al., 2007). Our study found that in the mice fed with the probiotic JB-1, depletion of Treg could lead to increased level of $Ly6C^{hi}$ monocytes. When we transferred the JB-1 induced Tregs into recipient mice, a reduction of $Ly6C^{hi}$ monocytes were found, and this reduction was not caused by simply the up-regulated number of Tregs, due to the truth that mice who received Tregs from water fed donors did not had altered level of $Ly6C^{hi}$ monocytes. It could be illustrated from these results that $Ly6C^{hi}$ monocytes might be one of the major targets of Tregs induced by probiotics, and limitation of the proliferation of activated monocytes is an important mechanism of immunosuppression in the homeostasis maintained by microbiota-gut-brain axis.

Overall, immunoregulation is an essential component of the microbiota-gut-brain axis and can protect the brain from stress induced behavioural changes. Our results suggest that probiotic supplementation, through induction of immunosuppressive $CD4^+CD25^+$ regulatory T cells, could limit the expansion of inflammation, mainly activated monocytes, and help the host to maintain homeostasis. Future directions could focus on the mechanism of the interaction between Tregs and monocytes, as well as exploration of possible immunosuppressive functions conducted by Tregs in the brain.

6.4. Vagus nerve ameliorates behavioral malfunction in mice by inhibition of microglial activation.

There is now strong evidence suggesting the integrity of vagal afferents is critical to communication between specific gut microbes and the brain. Bercik et al. found that the ability of *Bifidobacterium longum* to protect against the anxiety-like behaviour associated with dextran sodium sulfate (DSS) treatment was lost vagotomized animals (Bercik et al., 2011). Our lab's previous works demonstrated that vagotomy could block the anxiolytic and anti-depressive effects of *Lactobacillus rhamnosus* JB-1 in mice, and inhibited the changes on expressions of $GABA_A$ subunit in hippocampus induced by *L. rhamnosus* (Bravo et al., 2011a). Further studies identified that acute activation neurons in multiple brain regions following feeding with *L. rhamnousus* was also inhibited by vagotomy. Vagotomy was also demonstrated to attenuate the ability of *L. reuteri* rescued social deficits in models of autism spectrum disorder (ASD) (Sgritta et al., 2019). Another study demonstrated that administering cocktail of *L. helveticus* and *B. longum* to mice with myocardial infarction, reversed the increase in caspase activity in the amygdala, and subdiaphragmatic vagotomy abolished this effect (Malick et al., 2015).

In this thesis, we confirmed the findings of Bravo et al. that anxiolytic effects of oral JB-1 were absent in vagotomised mice. We also demonstrated for the first time that the ability of JB-1 treatment to supress the corticosterone increase following acute stress exposure was also dependent on an intact vagus nerve.

Corticosterone is the hormone of HPA axis, a neuro-endocrine system which plays vital in stress response, and can suppress inflammation quickly and systemically. The level of corticosterone usually rises shortly after exposure to stressful event, and drops back to normal level after a period of time. The dysregulation of HPA axis reactivity is commonly seen in animals with behavioral abnormalities. Myers et al. demonstrated that elevated corticosterone in the amygdala led to persistent increases in anxiety-like behavior and pain sensitivity (Myers et al., 2010). Also, exposure to high level of corticosterone increased depression-like behavior in rats (Ali et al., 2015; Kalynchuk et al., 2004). Importantly, HPA axis is also a part of microbiota-gut-brain axis (Sudo, 2012). Restraint stress led to higher level of corticosterone in germ-free mice, compared to mice with normal flora, indicating that a healthy gut microbiota contributes to the maintenance of HPA axis response (Sudo et al., 2004). Our current results showed that gut microbe communication with the HPA axis relied on the integrity of the vagus nerve.

Moreover, vagus nerve plays a vital role in immune modulation, and the loss of vagal integrity leads to enhanced severity of inflammation following immune challenge. In a clinical study, patients who had undergone vagotomy had elevated mortality and septicemia following physical trauma suggesting the vagus nerve contributes to the systemic response to injury (Peterson, Krzyzaniak, Coimbra, & Chang, 2012). Administration of dextran sodium sulfate (DSS), a reagent usually used to induce inflammatory bowel disease in animals, to vagotomized mice caused higher disease activity of colitis and increased inflammatory cytokines in

colon (J. E. Ghia, Blennerhassett, Kumar–Ondiveeran, Verdu, & Collins, 2006). In Chapter 5, we found that after vagotomy, there was significant increase of Ly6C^{hi} monocytes in periphery, suggesting a prolonged high inflammatory statue. Moreover, we also found that Tregs, as an immunosuppressive cell population, was not increased after JB-1 feeding in vagotomy group, indicating that the ability of promoting the Tregs' proliferation by JB-1 through microbiota-gut-brain axis also relied on an intact vagus nerve.

Maintenance of brain homeostasis and normal behaviors require a healthy regional immune environment along microbiota-gut-brain axis, there has been suggestions that vagotomy could change the signalling from the periphery immune system to the brain, thus affect CNS function indirectly. Intraperitoneal administration of IL-1β leads to reduced social exploration in rats, an effect that is attenuated by vagotomy, however, if IL-1β is administered through cerebral ventricle, vagotomy fails to inhibit the reduction of social exploration (Bluthe, Michaud, Kelley, & Dantzer, 1996), suggesting that vagus nerve helps to transmit the immune signal from periphery to the brain. Gallaher et al. demonstrated that subdiaphragmatic vagotomy in rats leads to a higher level of activated microglia in the hindbrain and nodose ganglia, with lower levels of microglia in the spinal cord, indicating the vagus nerve helps to inhibit inflammation in the CNS induced by microglia (Gallaher, Ryu, Herzog, Ritter, & Czaja, 2012). Inflammatory monocyte infiltration, as well as microglia activation in specific brain regions are demonstrated to be correlated with neuronal abnormality and behavioral alterations (Wohleb,

Terwilliger, Duman, & Duman, 2018). Microglia are important immune cells in brain, which are activated during brain infection and inflammation, respond to stress, and contribute to both pathogenesis or to inhibit neuronal protection and regeneration (Wake, Moorhouse, & Nabekura, 2011). Microglial activation is evident in mental conditions. In patients with depression, an increase in microglial activation and macrophage recruitment was found in post-mortem dorsal anterior cingulate matter (Torres-Platas et al., 2014). A correlation between microglial activation and suicide was observed in several brain regions of the depressive and schizophrenic patients, including dorsolateral prefrontal cortex, anterior cingulate cortex, hippocampus and mediodorsal thalamus (Steiner et al., 2008). Vagus nerve stimulation was found to be able to inhibit the microglial activation, and to protect against cerebral ischemia/reperfusion injury (Zhao, Zhao, Qin, Wan, & Fan, 2019). Decreased neuroinflammation, indicated by lower level of microglia, was correlated to higher vagus nerve activity fluctuations in near-term ovine fetuses, indicating the existence of the cholinergic anti-inflammatory pathway in brain, specifically targeting microglia (Frasch et al., 2016). In the current thesis, we found that microglia activation in hippocampus could be ameliorated after JB-1 administration, while vagotomy led to significant increase of Iba-1$^+$ microglia, with or without JB-1 treatment. These results are consistent with the behavioral and immune changes, indicating that an intact vagus nerve is required for gut microbe induced changes in the endocrine system and the immune system in the CNS and periphery that are associated with behavioral effects of the bacteria.

6.5. Conclusions

In the last decade we have seen a growing number of studies on the involvement of the microbiota-gut-brain axis in stress related psychiatric disorders. In this thesis, we present several novel findings regarding immune and neural modulation by the probiotic *Lactobacillus rhamnosus* JB-1. In Chapter 3, we have presented novel findings that early-stage intervention of JB-1 or sertraline after stress reduced hippocampal gene expression of molecules related to fear extinction, which is associated with persistence of fear memory and social behavior deficits. This study has implications, for behavioral and neural modulation by probiotics in relation to trauma exposure and PTSD. Additionally, the results suggest exploring the therapeutic possibility for probiotics or SSRI in disorders with memory deficits, for example, Alzheimer's disease. Moreover, we have explored the relationship between different components in the microbiota-gut-brain axis, as illustrated in Figure 1. To be specific, in Chapter 4, we have followed up our previous observations (Karimi et al., 2012) showing that the $CD4^+CD25^+$ regulatory T cells are an essential immune cell component in JB-1 induced homeostasis. We demonstrate that $CD4^+CD25^+$ cells, are both necessary and sufficient for *L. rhamnosus* JB-1 induced antidepressant and anxiolytic-like effects on the trait behaviour of male BALB/c mice. This further highlights the importance of the immune system in modulating behavior as well as the potential for immunotherapy in the management of psychiatric disorders. Although the downstream mechanism is unclear, a Treg driven reduction in activated monocytes may be involved.

However, we cannot exclude the possible action of Tregs by JB-1 in the brain, as changes in blood-brain barrier permeability have been shown to allow penetration of Tregs into brain to conduct immunoregulation directly (Pollak et al., 2018). In Chapter 5, we demonstrate, for the first time, loss of vagal integrity inhibits JB-1 induced modulation of the HPA axis response and the induction of regulatory T cells (Liu et al., 2020). In addition, subdiaphragmatic vagotomy alone causes an increase in activated splenic monocytes and hippocampal microglia. Thus, vagotomy disrupts multiple components of immune and endocrine changes that are associated with the anxiolytic effect of the bacteria. Future work should continue to decode the crosstalk between immune and nervous systems and how Tregs and vagus nerve work together to limit the neuroinflammation including activation of microglia/monocytes and how this is associated with behavioral changes. It may not be possible for people to avoid exposure to psychological stress, but with more understanding of the microbiota-gut-brain axis, we may be able to develop microbe-based approaches to reduce the prevalence and improve the prognosis for stress related disorders in the future.

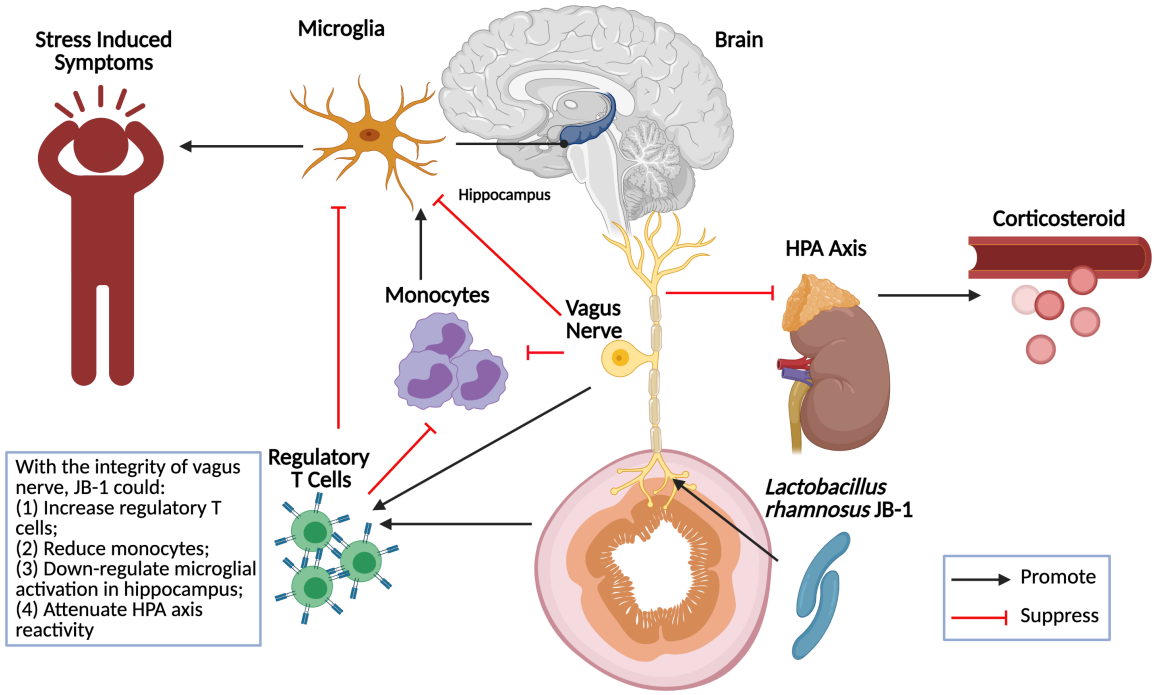

Figure 1. The vagus nerve and Tregs play vital roles in the maintenance of homeostasis along the microbiota-gut-brain axis.

CHAPTER 7. REFERENCES FOR INTRODUCTION AND DISCUSSION

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