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Research paper

# Neurobiological effects of a probiotic-supplemented diet in chronically stressed male Long-Evans rats: Evidence of enhanced resilience

Nick R. Natale [a,1], Molly Kent [b,1], Nathan Fox [a], Dylan Vavra [a], Kelly Lambert [a,*]

[a] Dept of Psychology, University of Richmond, VA 23173, USA
[b] Dept of Biology, Virginia Military Institute, Lexington, VA 24450, USA

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ABSTRACT

Probiotics that regulate the microbiome-gut-brain axis and provide mental health benefits to the host are referred to as psychobiotics. Preclinical studies have demonstrated psychobiotic effects on early life stress-induced anxiety- and depression-related behavior in rodents; however, the specific mechanisms remain ill-defined. In the current study, we investigated the effects of probiotic supplementation on neurobiological responses to chronic stress in adult male Long-Evans rats. Twenty-four rats were randomly assigned to probiotic (PB) or vehicle control (VEH) groups, then to either chronic unpredictable stress (CUS) or no-stress control (CON) conditions within each group (n = 6/subgroup). We hypothesized that PB supplementation would reduce markers of anxiety and enhance emotional resilience, especially in the CUS animals. In the cognitive uncertainty task, a nonsignificant trend was observed indicating that the PB-supplemented animals spent more time oriented toward the food reward than VEH animals. In the open-field task, CUS-PB animals spent more time in the center of the arena than CUS-VEH animals, an effect not observed between the two CON groups. In the swim task, the PB animals, regardless of stress assignment, exhibited increased floating, suggesting a conserved response in a challenging context. Focusing on the endocrine measures, higher dehydroepiandrosterone (DHEA)-to-corticosterone fecal metabolite ratios, a correlate of emotional resilience, were observed in PB animals. Further, PB animals exhibited reduced microglia immunoreactivity in the basolateral amygdala, possibly indicating a neuroprotective effect of PB supplements in this rodent model. These results provide evidence that PB supplementation interacts with stress exposure to influence adaptive responses associated with endocrine, neural, and behavioral indices of anxiety.

## 1. Introduction

When experienced on a chronic basis, psychological stress is considered a causal agent in debilitating psychiatric illnesses (Davis et al., 2017). Depression, for example, is a stress-related psychiatric condition that has been declared a leading cause of disability, affecting over 300 million people worldwide (Friedrich, 2017). The recent stagnation in the development of effective psychopharmacological treatments for psychiatric illnesses highlights the need for innovative approaches to target these unmet medical needs (Hyman, 2012; Hyman, 2014). In recent years, a correlation between intestinal microbiota composition imbalance (dysbiosis) and neuropsychiatric disorders was established, notably in patients with major depressive disorder (Capuco et al., 2020). Consequently, the correction of dysbiosis has been introduced as a novel potential therapeutic approach for various mental illnesses (Liu, 2017) as accumulating evidence indicates that the impact of the microbiota extends to the nervous system, modulating behavior via endocrine and immune factors influenced by the microbiota-gut-brain axis (MGBA) (Cryan and Dinan, 2012; Dinan and Cryan, 2016; Foster and McVey Neufeld, 2013; Leung and Thuret, 2015; Sarkar et al., 2016). Hence, the complex ecosystem of the GI tract provides a valuable resource for the exploration of physiological mechanisms regulating the MGBA that contribute to emotional resilience and other measures of well-being (Kho and Lal, 2018).

Relevant to stress-related disorders, there is also convincing evidence that the gut-brain axis is an influential factor in the hypothalamic-pituitary-adrenal (HPA) stress response and has been referred to as a "master regulator" of the stress response system (Dinan and Cryan,

* Corresponding author at: Dept of Psychology, B327 Gottwald Science Center, University of Richmond, VA 23173, USA.
*E-mail address:* klambert@richmond.edu (K. Lambert).
[1] Co-first authors.

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2016). Given that elevated cortisol is correlated with depression symptoms in humans, the role of dysbiosis in the modulation of the stress response, a question of interest in the current study, may yield valuable information about building resilience against emerging stress-related psychiatric illnesses such as mood disorders (Dienes et al., 2013; Zobel et al., 2001). Interestingly, increased levels of the adrenal steroid hormone dehydroepiandrosterone (DHEA) in proportion to corticosterone levels have been associated with enhanced resilience against the emergence of negative health effects associated with high corticosterone levels (Morgan et al., 2009). An example of the intricate interaction between the enteric and central nervous systems is highlighted in irritable bowel syndrome (IBS; (Drossman, 2006), a common stress-related condition with global prevalence rates approaching 11% (Lovell and Ford, 2012). IBS is characterized by recurrent abdominal distress that is related to HPA axis functions. Although a single microbial contributing factor has yet to be identified in IBS, host-microbe interactions are suspected in this stress disorder (Moser et al., 2018).

Microbes associated with positive health effects, known as probiotics, have been administered in preclinical and clinical research contexts (Vlasova et al., 2016), with some shown to exert positive effects on mental health (Bercik et al., 2011; Park et al., 2011). Notably, the probiotic supplement used in the current study has been shown to mitigate the pervasive effects of early-life stress on anxiety- and depression-related behavior, HPA axis activity, and pubertal timing in mice (Gareau et al., 2007; Cowan et al., 2016; Cowan et al., 2019a; Cowan and Richardson, 2019b; Peng et al., 2019). Further, these effects may employ epigenetic mechanisms and persist as transgenerational effects in subsequent generations (Callaghan et al., 2016). Psychobiotics, which include probiotics with documented impact on CNS functions and related behaviors, modulate the microbiota-gut-brain axis and are viewed as a promising therapeutic approach for psychiatric illness (Cheng et al., 2019; Dinan and Cryan, 2016; Sarkar et al., 2016; Cheng et al., 2019; Bermúdez-Humarán et al., 2019). One mechanism by which microbes and their metabolites alter the transport of neuroactive chemicals into the CNS is through increased permeability of the gut wall, allowing psychoactive chemicals to enter the blood where they are subsequently transported to the brain (Cani et al., 2016; Cani and Everard, 2016). Further, microbiota modify levels of pro-inflammatory and anti-inflammatory cytokines in the blood, both of which can indirectly impact brain function (Cryan and Dinan, 2012). Although the extent of control the microbiota have in the host nervous system remains unclear, they likely guide behavior through modulation of neurochemical systems (Johnson and Foster, 2018).

Extending beyond the recognition that neurochemicals are altered by probiotics, neuroanatomical mechanisms have also been explored. Consisting of approximately 80% afferent nerve fibers (Bonaz et al., 2018), the vagus nerve transmits valuable sensory information related to the heart, lungs, and gastrointestinal tract to the brain (Cryan and Dinan, 2012) and it is through this vagus nerve pathway that many neurochemicals and brain functions are modulated by the microbiota (Sampson & Mazmanian, 2015; Bercik et al., 2011; Perez-Burgos et al., 2013). The vagus nerve, however, is unlikely to be the only mediator of microbiome-influenced neurobiological processes. Interestingly, while some effects of probiotics on behavior and brain molecular physiology are negated by vagotomy (Bercik, 2011; Bravo 2011; Malick 2015), microbiota have been shown to modulate brain-derived neurotrophic factor (BDNF) levels in the hippocampus of mice following vagotomy surgery (Bercik et al., 2011). Research with germ-free mice indicates that the disrupted gut ecosystem in these animals results in an exaggerated corticosterone response to acute stress; a response that was reversed by the administration of *Bifidobacterium infantis* (Luczynski et al., 2016; Sudo et al., 2004). Further, focusing on stress-related behavioral responses, germ-free rats exhibit increased anxiogenic behavior in an open-field task, accompanied by a 2.8 fold increase in corticosterone levels, as well as increased mRNA levels of corticotropin-releasing factor in the hypothalamus and glucocorticoid receptors in the hippocampus (Crumeyrolle-Arias et al., 2014).

Of interest in the current study are the potential immunological and neural mechanisms contributing to microbiome-influenced effects that are representative of stress responsivity and depression-like symptoms in chronically stressed animals. BDNF is abundantly expressed in the CNS and is viewed as an important mediator of neuroplasticity and neuroimmune functions that are dysregulated in depressed patients (Jin et al., 2019; Lee and Kim, 2010; Li et al., 2018; McGinty et al., 2010; Yu and Chen, 2011). Because BDNF fluctuates in response to pro-inflammatory markers in an inverse fashion, this neurotrophic factor has been referred to as a potential bridge between inflammation and neuroplasticity (Calabrese et al., 2014). For example, patients with inflammation are known to exhibit comorbid depression symptoms (Benton et al., 2007b; Benton et al., 2007a; Redlich et al., 2018), and animals exposed to immunological threats exhibit depressive-like phenotypes (Frenois et al., 2007). Further, glucocorticoids, also involved in depression phenotypes, are known to suppress BDNF levels (Daskalakis et al., 2015; Yehuda and Daskalakis, 2015). Resident macrophages of the central nervous system known as microglia represent an additional mediating mechanism between the gut microbiome and neural processes (Wang et al., 2016, 2018). Some probiotics have been shown to buffer proinflammatory microglial phenotypes (Chunchai et al., 2018) and enhance neuroplasticity in rodents exposed to a chronic high fat diet and associated inflammation (Buffington et al., 2016; Myles et al., 2020a, 2020b). Since disrupted neural-microglia interactions have been associated with anxiety disorders such as depression, this may represent a mechanism for the antidepressive effect of some probiotics (Wohleb, 2016). Due to the putative role of the basolateral amygdala in the mediation of peripheral markers of emotional and stressful responses, microglia have been investigated in this brain area (Munshi et al., 2020). Additionally, compromised microglial responses have been observed in germ-free mice due to the role of microbiota in microglia maturation and neurodevelopment (Erny et al., 2015, 2017; Thion et al., 2018). Although the specific roles have yet to be identified, these microbiome-microglia interactions have also been implicated in neurodevelopmental conditions such as Autism Spectrum Disorder (Lammert et al., 2018; Sharon et al., 2019; Vuong and Hsiao, 2017) and aging-related cognitive impairments (Boehme et al., 2021), suggesting that gut-microbe microglia modifications play a role in psychiatric and neurodevelopmental diseases beyond depression.

Considering the building evidence that the gut microbiome modulates brain function via multiple mechanisms including altered neuroplasticity and neuroimmunological functions, especially in stressed animals (Papalini et al., 2018), the current study investigated the impact of a probiotic-supplemented diet on targeted behavioral, neural, and endocrine markers of various stress and anxiety responses. Of particular interest is the influence of probiotic supplements on the chronic stress response in order to enhance the translational value to humans experiencing long-term stressors. Specifically, male rats receiving either a probiotic formulation (*L. helveticus* R0052 and *L. rhamnosus* R0011) or a vehicle control supplement were exposed to chronic unpredictable stress and subsequently assessed for anxiogenic responses in two behavioral tasks. Following these assessments, HPA axis activity was evaluated by evaluating corticosterone and DHEA fecal metabolites, endocrine markers of stress, and emotional resilience, respectively (Bardi et al., 2010, 2012; Kent et al., 2017). Additionally, immunohistology was used to assess neural BDNF levels and microglia activation. Several relevant dependent variables were included in this study to provide a multidimensional perspective of the interdependent relationship among relevant neural, endocrine and behavioral variables. Based on previously reported findings, we hypothesized that rats consuming the probiotic formulation would exhibit increased behavioral and neurobiological markers of emotional resilience.

## 2. Materials and methods

### 2.1. Animals

All experimental procedures were conducted in accordance with the University of Richmond Institutional Animal Care and Use Committee and Institutional Review Board. Twenty-four male Long-Evans five-week-old rats, weighing between 115 and 200 g, were obtained from Envigo (Indianapolis, Indiana, USA). Upon arrival, rats were handled daily for one week to habituate them to the researchers. The animals received standard rodent chow and water ad libitum throughout the experiment. To monitor water consumption rates, all rats were single-housed under a 12/12 light-dark cycle with lights on at 6:00 am. Room temperature was kept constant at 22 °C. Body weights and water consumption volumes were assessed daily. The rats were randomly assigned to one of two stress treatment groups, chronic unpredictable stress (CUS) or no stress control (CON), with half of the animals from each group further assigned to either a regimen of water supplemented with the probiotic (PB) or only vehicle (VEH) which contained comparable amounts of maltodextrin and milk powder (n = 6 each group; see Fig. 1 for group assignments and timeline for the project).

### 2.2. Probiotic formula administration

A commercially available probiotic (PB) combination of *Lactobacillus helveticus* R0052 and *Lacticaseibacillus rhamnosus* R0011, Lacidofil®, was generously supplied by Lallemand Health Solutions, Inc. (Mirabel, Canada). For 27 days beginning one week after their arrival, the rats were given two hours prior to the beginning of the dark cycle each day to drink the PB-infused water or vehicle control preparation. This duration was deemed appropriate for animals to consume sufficient fluid and minimized the time for potential spillage that would inflate consumption amounts. The rats were given unaltered water for ad libitum consumption during the remaining 22 h each day. The PB-infused water was prepared by rehydrating 2.87 g of powder (mixed with maltodextrin and milk powder) in 75 mL of distilled water for a final concentration of $10^9$ CFU/mL. The vehicle control (VEH) solution was prepared by rehydrating 2.87 g of maltodextrin/milk mixture in 75 mL of distilled water. Thus both the PB and VEH animals received the same Maltodextrin/milk formula each day with the PB animals also receiving the PB mixed in the preparation. Specifically, this combination included 95% L. *rhamnosus* R0011 and 5% L. *helveticus* R0052. PB and VEH treated animals were housed in separate cages, on different racks within the same room. This Lacidofil formula has been shown to effectively alter microbiome-affected responses in past studies (e.g., Gareau et al., 2007). Across the exposure phase, there was no difference in average daily consumption of the two formulas between the CUS and CON groups ($t_{22}$ =0.181, p = 0.858; mean±SEM: VEH=8.68 mL±0.55; PB=8.81 mL±0.49). There was also no difference in the amount of daily water consumed ($t_{22}$ =0.79, p = 0.438; mean±SEM: VEH=10.6 mL±0.56; PB=11.2 mL±0.5; additionally there was no effect of PB-supplements on body weight (taken during the behavioral assessments); however, a main effect for stress was found [Stress/CUS= 231.48 (+15.57) and CON = 247.94 (+8.56); F(3,23)= 9.699, p = 0.005 partial eta 0.327].

### 2.3. Chronic unpredictable stress protocol

Following a week of PB administration, rats assigned to the chronic unpredictable stress (CUS) group were housed in a separate room from the rest of the colony to avoid indirectly exposing animals to stress-related stimuli. Exposure to ecologically relevant stressors occurred twice daily at unpredictable times for 13 days. The stressors consisted of both environmental-based (e.g., altered sounds, bedding, and lights) and survival-based components (e.g., predator odor, swim exposure; see Table 1). Fecal samples were collected prior to the commencement of the CUS protocol (baseline) and during the CUS exposure (midway and end of CUS period) for endocrine assays. After approximately 13 days on the designated diet combined with stress exposure, all animals were assessed in two anxiety-related behavioral tasks specifically, decision-making in the uncertainty challenge task (a task our lab developed to

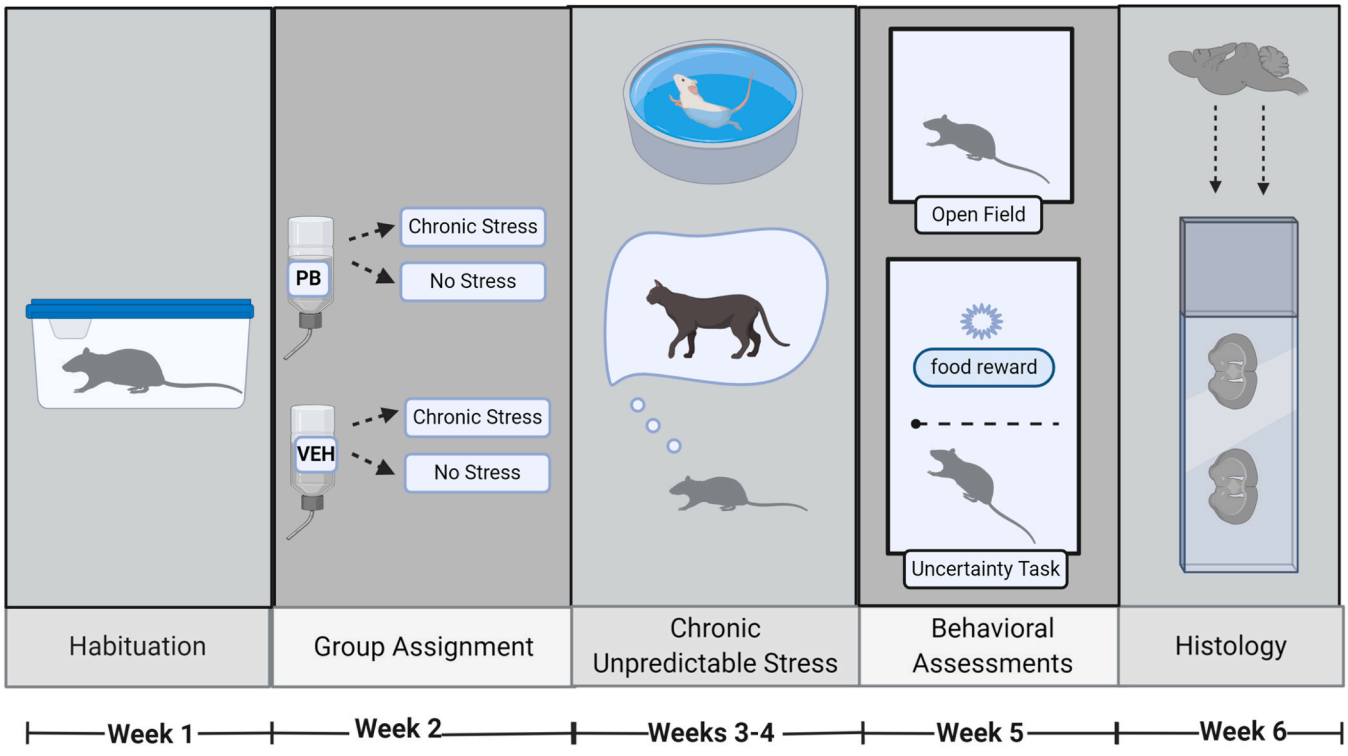


**Fig. 1.** Group assignments and timeline of project phases. Diet treatment (VEH and PB) continued throughout CUS and behavioral testing. CUS lasted for 13 days; following stress exposure, behavioral tasks were performed. During behavioral assessments, each test was separated by at least 24 h.

**Table 1**
Categorization of stressors in chronic unpredictable stress paradigm.

| Environmental stressors | Survival-based stressors |
|---|---|
| Wet bedding (overnight) | Soiled cat litter (overnight) |
| Strobe light (4–6 h) | Fox urine (overnight) |
| Tilted cages (2–4 h) | Predator noise (2–4 h) |
| Small plastic clip on tail (<30 min) | Forced swim (5 min) |

investigate food motivation in the presence of a novel barrier; see Scarola et al., 2020) and exploration in the open-field task.

### 2.4. Uncertainty challenge task

During the uncertainty challenge task, animals were first placed in a 1.5 × 6 x 0.5-meter arena (Fig. 2) with bedding distributed on the floor and a food reward (piece of Froot Loop cereal) available for consumption in the area of the arena designated as the reward zone (see Scarola et al., 2020). During this initial trial, the rat was individually placed in the arena for five minutes to explore and consume the food reward. If the food reward was eaten prior to the 5-minute period, the animal was removed so it wouldn't be exposed to the reward zone without a food reward. A hanging partition with metal chains and bells was placed in the center of this arena; however, during the initial habituation trial, the partition was pulled back so that the animal had access to the entire area with no physical barriers. Prior to habituation training in this task, all rats were exposed to a cereal treat in their home cages so they were motivated to eat the food reward. On the day of testing, food was removed from the animals' home cages three hours before the session so they would be food motivated. During the test trial, the partition was positioned to create a barrier between the start and reward zones so that the rat could not see the other side of the apparatus where the food reward was located. Because the area on the other side of the partition (i.e., the previously experienced reward zone) was not visible to the rats, a decision to pass through the curtain to the food reward zone was viewed as a bolder contextual assessment than the animals that remained in the safe zone of the task. Thus, animals made a decision to either stay in the familiar safe zone or enter the uncertain reward zone on the other side of the partition. During this single-trial test, latency to approach the novel partition, latency to pass through the partition, latency to eat the cereal treat, and percent of time spent oriented toward and in proximity to the food reward were recorded by a Noldus computerized tracking system. Similar to the habituation trial, the duration of the test trial was five minutes (or until the food reward was consumed). Upon the return to their home-cages, animals were once again given an ad libitum diet.

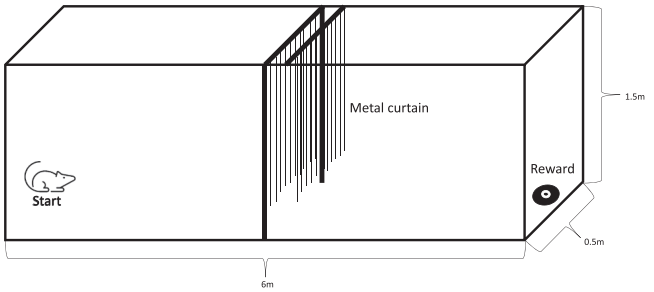


**Fig. 2.** *Cognitive Uncertainty Task.* Graphical depiction of the uncertainty challenge task. Each animal was placed in the start location and required to cross the metal barrier to retrieve the food reward on the opposite side. Zones were created in Noldus to measure latency to cross the barrier and approach the food reward. Duration spent near the start location, near the barrier, and near the food reward were also measured.

### 2.5. Open field test

Following two weeks of CUS, the animals were placed in an open arena (1 × 1 m) for five minutes and both a human observer and computerized tracking system (Noldus) monitored and recorded their exploration/behavior. Each rat was given an initial habituation to the arena followed by a second trial to assess behavior in each session. The second trial occurred 24 h after the first. For both trials, the location and movement of the rat in the open field was monitored via the tracking software with the center of the arena representing approximately 10% of the total area of the open field. Additionally, the following behaviors were quantified by an observer blind to the conditions: freezing (defined as two seconds of inactivity not appearing to be resting or sleeping), grooming, internal rearing (i.e, rearing directed toward the internal area of the arena), escape attempts (i.e., rearing or climbing responses directed toward the walls of the apparatus), and digging (two or more paw-strokes in the bedding).

### 2.6. Swim test

For two consecutive days during the CUS exposure, the chronic unpredictable stress rats were placed in a 91 × 45 x 40 cm water tank for five minutes as part of their stress exposure. For each trial, an observer blind to group conditions recorded the frequency of the following behaviors: floating, diving, and half-dives (defined as submerging the head underwater but not traveling to the bottom half of the tank). Latency to swim and duration of time spent floating were also recorded. Twelve hours after each forced swim test, fecal samples were obtained to assess stress hormone levels during the swim task and placed in a −80 freezer (see Bardi et al., 2010; Kent et al., 2018).

### 2.7. Histological preparation

Following the behavioral tests, all animals were anesthetized and perfused so the brains could be harvested for histological assessment (see Kent et al., 2018). Specifically, rats were individually anesthetized with isoflurane inhalation and closely monitored until they were sufficiently nonresponsive. At this time, they were perfused transcardially at 40 mL/min using a MasterFlex L/S perfusion pump initially with 100 mL of PBS, then with 200 mL of 4% paraformaldehyde. Each brain was subsequently placed in a container with 4% paraformaldehyde overnight at 4 °C. The following day, the brains were transferred to a 10% sucrose solution and sequentially moved into a 30% sucrose solution at 4 °C. The brains remained in the sucrose solution until sectioning with a cryostat at −25 °C. For each brain, six sections (40 μm thickness) were obtained through the basolateral amygdala and the dorsal hippocampus. To avoid double-counting of individual cells, every third consecutive section was used, allowing for a 120-micron distance between each section analyzed.

### 2.8. Immunohistology and neural quantification

Following sectioning, brain sections were immunostained for brain-derived neurotrophic factor (BDNF) and microglia visualization. Specifically, brain sections were incubated overnight with the BDNF primary antibody [1:1000 Rabbit polyclonal (Bioss bs-4989R; Woburn, MA)] diluted in a solution of PBS-BT + 1% NGS, followed by 1.5-hour incubation with secondary antibody (1:250 goat anti-rabbit (Vector; Burlingame, CA) diluted in a solution of PBS + 1% NGS. In addition, the brain sections designated for microglia assessment were incubated overnight with the Iba1 antibody [1:10000 (Fujifilm Wako Chemicals 019–19741; Richmond, VA)] that targets microglia and biotinylated secondary antibodies [1:250 dilution; goat anti-rabbit; Vector Laboratories (Burlington, CA)]. Following primary and secondary antibody steps, all brain sections were exposed to an Avidin-Biotin Complex (Vector). All brain sections were then stained with a DAB peroxidase

substrate (30% $H_2O_2$ + 0.6% Tris buffer + 0.3% $NH_3$Nis + 0.02% DAB, Vector) and cleared through a series of 70%, 95%, and 100% EtOh and Citrisolv washes prior to being coverslipped with Permount mounting medium (Electron Microscopy Sciences; Hatfield, PA).

BDNF immunoreactive cells were quantified in the hilus and CA3 regions of the hippocampus with a Zeiss Axioskop light microscope (Carl Zeiss, Oberkochen, Germany) and Neurolucida software (Microbrightfield, Inc., Williston, VT). Microglia immunoreactive cells were quantified via light thresholding in the basolateral nucleus of the amygdala (BLA) with Bioquant software (Bioquant, Nashville, TN); this technique allowed us to determine the percent of immunoreactive tissue in proportion to the area of tissue in the full visual field. For each of the antibodies, six visual fields were used for analysis for each animal.

### 2.9. Corticosterone & dehydroepiandrosterone (DHEA) quantification

A baseline fecal sample was obtained from each rat the day before the commencement of the CUS protocol and after each of the two swim stressors (for CUS animals); samples from CON animals were also collected at these time points. Fecal samples were collected at 9:00 am across all sampling time points by placing rats in a cage with no bedding and retrieving the bolus that was typically produced with approximately five minutes. The rodents were then returned to their home cages. Samples were placed in a labeled centrifuge tube and stored at −80 °C. An estimated 0.09 g of feces was removed from each fecal sample and placed into 1 mL of methanol, agitated and then centrifuged for 15 min. Using the assay procedures provided by an Enzyme ImmunoAssay (EIA) kit (Enzo Life Sciences, Farmingdale, NY), duplicate samples were prepared and transferred to the microplate. An automated microplate reader (BioTek, Winooski, VT, model Synergy) and Gen5 software (BioTek, Winooski, VT, version 2.04.11) were used to determine the hormone concentrations of corticosterone and DHEA in each sample. Readings were assessed at a wavelength of 405λ. The CORT assay had a sensitivity of 27 pg/mL with a range between 32 and 20,000 pg/mL. There is a cross-reactivity of less than one percent for progesterone (0.046%), testosterone (0.31%), tetrahydrocorticosterone (0.28%) aldosterone (0.18%), and cortisol (0.046%). The DHEA assay had a sensitivity of 2.9 pg/mL and a range between 12.21 and 50000 pg/mL. The EIA had a crossreactivity of 21.3% with 11-deoxycorticosterone and 21% with desoxycorticosterone. There is a cross-reactivity of less than one percent for progesterone (0.06%), testosterone (0.1%), aldosterone (0.29%), and cortisol (<0.02%). The standard curves for the hormone plates were only included if the $R^2$ value was greater than 95%. Intra- and inter-assay coefficients of variations were 2.26% and 9.15% for CORT and 2.22% and 7.45% for DHEA. For the duplicate samples, a coefficient of variation of 15% or less was considered acceptable for the current study.

### 2.10. Statistical analysis

Data were analyzed in SPSS (v. 26) and visualized with GraphPad Prism 8. The uncertainty challenge task, BDNF, and microglia data were analyzed with a two-way analysis of variance (ANOVA). A 2x2x3 mixed analysis of variance (Probiotic x Stress x Time) was used to analyze the hormone data, while a 2x2x2 mixed ANOVA was used to analyze the Open field test. A 2 × 2 mixed ANOVA was used to analyze the swim test data in the CUS animals. For all analyses, a P value of less than 0.05 was necessary for a significant effect; Tukey's post-hoc tests were used when necessary.

## 3. Results

### 3.1. Uncertainty challenge task

A 2 × 2 ANOVA revealed a nonsignificanttrend for dietary treatment (PB and VEH) for percentage of time the animal's nose was in proximity (4 cm) to the food reward in this task (a measure of targeted interest in the food reward). Specifically, the PB-treated groups spent a greater percentage of time near the food reward compared to the VEH groups ($F_{1,23}$)= 4.204, p = 0.054, $\eta_p^2$ = 0.174). (Fig. 3). No significant effects were observed for the other behaviors assessed in the cognitive uncertainty task; see Supplementary Table 1.

### 3.2. Open field task

A 2x2x2 mixed ANOVA (PB treatment x Stress x Time) revealed a significant effect of time on the number of seconds spent in the center of the arena ($F_{1,20}$ = 7.341, p = 0.013, $\eta_p^2$ = 0.268). Specifically, regardless of dietary treatment group, animals spent more time in the center of the arena in the second assessment. Additionally, a significant between-subjects interaction between PB treatment and stress exposure ($F_{1,20}$ = 4.863, p = 0.039 $\eta_p^2$ =0.196) was observed for number of seconds spent in the center of the arena for each time point. As seen in Fig. 4A, when the data are collapsed across both time points, in the CUS condition, the PB animals spent more time in the center than the VEH-controls, whereas in the no-stress CON condition, the VEH-control group spent more time in the center. Internal rearing responses, viewed as exploration responses, during the open field task were influenced by a significant interaction between time and PB treatment ($F_{1,20}$ = 8.464, p = 0.009, $\eta_p^2$ = 0.297). Specifically, PB-treated animals increased rearing from Trial 1 to Trial 2, but no change in VEH control animals was observed across trials (see Fig. 4B). No significant differences were observed for the remaining behaviors assessed in the open-field task (Supplimentary Table 1).

### 3.3. Forced swim task

For the CUS animals, a two-way mixed-design ANOVA (Probiotic x Time) indicated a nonsignificant trend for a between-subjects effect of dietary treatment ($F_{1,10}$ =3.966, p = 0.074, $\eta_p^2$ =0.284). Specifically, post hoc comparisons indicated that the trend for the main effect was seen in Trial 1, with PB-treated animals exhibiting a longer duration of floating than their vehicle-control counterparts (p = 0.048; See Fig. 5). None of the other behaviors indicated a significant difference between groups or across time points (Supplementary Table 1).

### 3.4. Hormone analysis

A 3-way mixed ANOVA of the hormone data revealed a significant

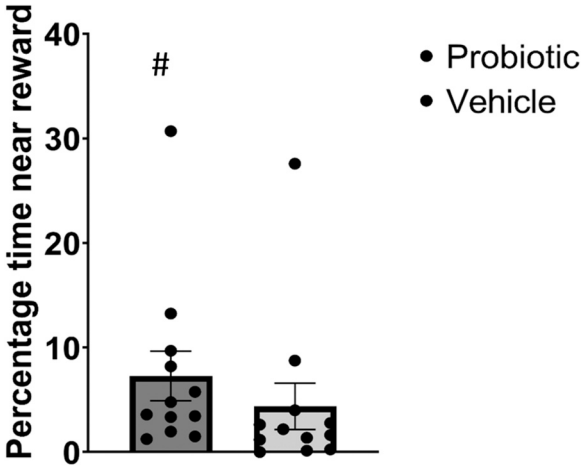


**Fig. 3.** *Cognitive Uncertainty Task*. Time spent in proximity to food reward during the test phase of the uncertainty task (n = 12 per group). Data expressed as mean (±SEM) percentage of time the nose of the animals spent in the zone surrounding the reward. #P = 0.054 for Probiotic and VEH groups.

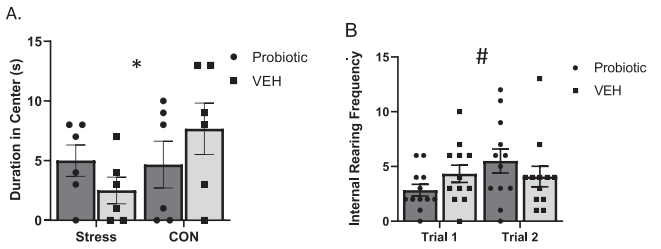


**Fig. 4.** *Open Field Task*. **(A)** Percent of time spent in the center of the open field arena (n = 6 per group) depicting the significant interaction. Data expressed as mean (±SEM) number of seconds. *P < 0.05 for interaction effect between stress and PB supplement. **(B)** Frequency of internal rearing responses in the open field arena (n = 6 per group). Data expressed as mean ( ± SEM) number of internal rears. #P < 0.01 for interaction effect.

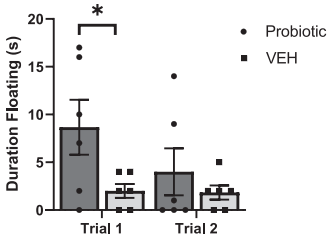


**Fig. 5.** *Swim Task*. Floating duration in the 2 trials of the swim test of chronically-stressed rats in the PB-supplemented (n = 6) and VEH (n = 6) cohorts. Data expressed as mean (±SEM) number of cumulative seconds floating *P < 0.05 for PB vs. VEH in Trial 1.

effect of time (baseline, mid-stress and late-stress) on CORT fecal metabolites ($F_{2,40}$ =4.482, p = 0.018, $\eta_p^2$ =0.183). Interestingly, CORT hormone levels were highest at baseline and decreased over time. As indicated in Fig. 6A, the stressed animals had higher CORT levels at each time point, although the differences weren't statistically significant. Analysis of DHEA indicated no difference in levels over time (Table 3). Focusing on the ratio of DHEA/CORT, a nonsignificant trend for an interaction among the variables of time, dietary treatment, and stress was observed ($F_{2,40}$ =3.066, p = 0.058, $\eta_p^2$ =0.133). Further planned analyses based on a priori assumptions suggested that after the first stress assessment, a main effect existed for each treatment variable in the DHEA/CORT ratios (see Fig. 6B). Specifically, a post-hoc analysis indicated that PB-treated animals had a significantly higher ratio than VEH-treated rats ($F_{3,23}$ = 4.357, p = 0.05, $\eta_p^2$ =0.179). Additionally, the no-stress control group had a significantly higher DHEA/CORT ratio compared to the stress group ($F_{3,23}$ = 7.259, p = 0.014, $\eta_p^2$ = 0.266).

### 3.5. Neural quantification

A 2 × 2 (Probiotic x Stress) ANOVA revealed a significant effect of PB treatment on microglia activation; specifically, PB-treated animals had less immunoreactive tissue in the basolateral amygdala than the VEH-control animals ($F_{1,23}$ =8.614, p = 0.008, $\eta_p^2$ = 0.301; see Fig. 7). Neural analysis did not reveal significant differences in BDNF levels assessed by immunostaining of the hippocampus sections (Supplimentary Table 2).

## 4. Discussion

Of interest in the current study was the impact of a probiotic supplement on behavioral and neurobiological responses to chronic unpredictable stress in rats. Considering that psychological stress generates a constellation of responses that are consistent with a depressive phenotype, it is important to understand factors that interact with the stress response from multiple neurobiological perspectives Further, understanding individual responses to health threats is critical in the analysis of psychiatric illnesses and may be especially relevant for conditions such as depression for which therapeutic progress has been much slower than other medical conditions such as diabetes or cardiovascular diseases (Dobbs, 2017; Insel, 2009).

Whereas acute stress is often investigated in preclinical rodent studies, the extended duration of the chronic unpredictable stress paradigm in this study provided an opportunity to investigate the effects of PB supplements in chronically challenged animals. Since past research suggests that the effects of PB-supplemented diets are most influential in stressful conditions (Papalini et al., 2018), the animals in this study were exposed to chronic unpredictable stress (Kent et al., 2018), as this is a preclinical model with putative translational value for human stress-related illnesses including depression (Geng et al., 2020; Hariri and Holmes, 2015; Nikolova et al., 2018).

In the anxiety-related behavioral tasks, a nonsignificant trend indicated that PB-supplemented animals spent more time in proximity and

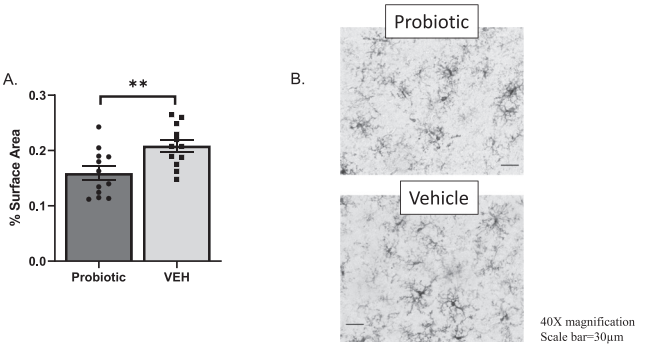


**Fig. 7. (A)** *Microglia immunoreactivity*. Percent area of Iba1 + tissue in basolateral amygdala 40 µ brain sections extracted from PB-supplemented (n = 12) and VEH-control (n = 12) rats. Data expressed as mean ( ± SEM) percentage of surface area exhibiting immunoreactivity. * *P < 0.01 for PB vs. VEH group. **(B)** Representative photomicrographs of microglia (Iba-1 + tissue) in the targeted area of the basolateral amygdala in the PB and VEH groups.

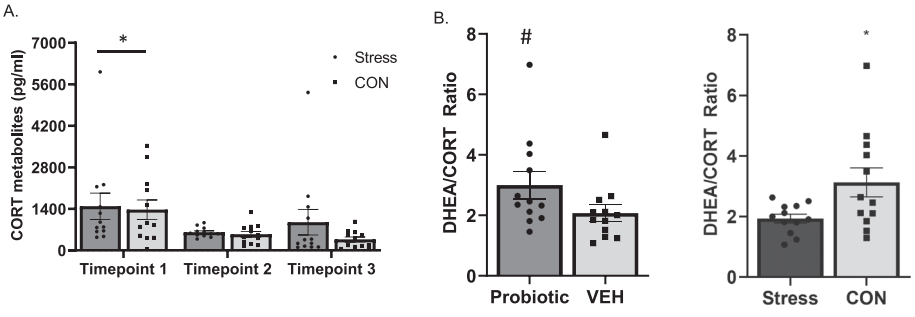


**Fig. 6.** *Endocrine Assays*. **(A)** Average Stress and CON corticosterone (CORT) levels in fecal samples across all 3 timepoints (N = 12 per stress group). Data expressed as mean (±SEM) CORT levels (pg/mL). *P < 0.05 for Timepoint 1 vs 2 and 3. **(B)** The ratio of DHEA to CORT in fecal samples of probiotic supplemented and VEH rats (n = 12 per group) Data expressed as mean ( ± SEM) DHEA/CORT ratio. #P = 0.051 for Probiotic vs. VEH in Swim Trial 1. **(C)** The ratio of DHEA to CORT in fecal samples of stressed and no stress (CON) rats. *P < 0.05 in Swim Trial 1.

oriented toward the reward, regardless of their stress exposure. Because the PB animals did not consume more food rewards in the task, it is likely that the PB animals were exhibiting heightened wariness in the context of food placement in a novel context (Koizumi et al., 2018), a potential explanation that requires further investigation. In the open field task, PB-supplemented animals exhibited increased exploration in the center of the arena compared to the stressed animals, although this effect of PB-supplementation was not observed in the non-stressed animals. Interestingly, for the rearing responses, the PB-supplemented animals increased this response from the first to the second trial, whereas this effect wasn't observed in the VEH animals. In this context, internal rearing responses are viewed as a behavioral measure of heightened exploration as an additional perspective is gained by observing from the higher position. In the swim task, PB-treated animals exhibited increased floating, often interpreted as a conserved response (de Kloet and Molendijk, 2016; Molendijk and de Kloet, 2015), but then reduced their floating duration between the first and second trials, an effect not observed in the VEH-control animals.

Considering all behavioral assessments in the current study, the results indicate that the PB-treated animals exhibited behavioral evidence of emotional resilience, especially in the stress condition. For example, in the CUS condition, PB animals exhibited a trend toward an increased motivation to acquire a food reward, increased exploration of an unfamiliar territory, and flexibility in their responses observed in the inescapable swim task. Although these effects are of interest, the lack of significant effects in many responses such as latency to cross the barrier in the uncertainty task suggest that the PB effects are not pervasive in the animals' stress and anxiety neurobiological responses, requiring further information to assess the role of PB supplementation as a preventative approach in stress-related psychiatric illness.

Past research with chronic paradigms has generated interesting insights about accompanying alterations in the microbiome. For example, mice experiencing four weeks of sleep fragmentation exhibited increased food consumption and changes in the gut microbiota characterized by increased growth of highly fermented species (i.e., *Lachnospiraceae* and *Ruminococcaceae* species and decreased evidence of *Lactobacillaceae* families) that were associated with adipose tissue inflammation and altered insulin sensitivity (Poroyko et al., 2016). Models of chronic social stress have also altered gut microbiome populations, with initial agonistic encounters exerting a differential effect on dominant and subordinate hamsters (Partrick et al., 2018). The impact of PB-supplemented diets on the prevention of health-compromising allostatic load during the chronic unpredictable stress model in mice recently demonstrated that reproductive-associated effects of stress exposure (e.g., sperm deficits) were reversed by the administration of *L. rhamnosus* (Guo et al., 2020). Further, the behavioral phenotype associated with behavioral despair was accompanied by compromised *Lactobacillus*, and the depressive-like behavior was reversed with *Lactobacillus* diet supplementation (Marin et al., 2017).

Focusing on biological markers of emotional resilience, although dietary treatment or stress-exposure did not affect BDNF levels, microglia activation was reduced in the PB-supplemented animals; specifically, the PB-treated animals exhibited less immunoreactivity to the microglia activation marker Iba1 in the basolateral amygdala than the vehicle control animals. Further, the endocrine data pointed to higher DHEA/Corticosterone ratios in the PB-treated animals, an effect associated with less severe stress responsivity, following the initial swim exposure (Lambert et al., 2014). An interesting observation with the endocrine data, however, was the higher baseline corticosterone levels than those observed at the subsequent time points. The higher levels may be due to the stress of settling into the new lab conditions at the beginning of the study. The absence of a PB effect on baseline measures before the animals were exposed to stress, however, suggest that probiotics may be more advantageous in psychologically challenging circumstances than in less stressful conditions (Papalini et al., 2018). These results corroborate recent findings indicating that Lactobacillus paracasei H1101 decreased the salivary CORT/DHEA metabolites in humans (Lalitsuradej et al., 2020), suggesting that the results have potential for translational relevance. Thus, the current endocrine findings provide additional support that the gut microbiome may play a regulatory role in the stress response (Karl et al., 2018), providing a potential therapeutic window for the treatment of stress-related psychiatric illnesses.

With building evidence of the cross-talk between stress and immune systems, the role of microglia, known as neural immune cells (Bellavance and Rivest, 2014; Manley et al., 2018), in gut microbiome-mediated chronic stress responses, was also of interest in this study. Given our findings that PB-supplemented animals exhibited less microglia immunoreactivity in the basolateral amygdala, these results provide further support of microglial-microbiota interactions (Rea et al., 2016). The self-renewal and plasticity of the microglial cells are undoubtedly advantageous for an agile and responsive neural response to stress that may be further mediated by the gut microbiome (Abdel-Haq et al., 2019). The absence of PB influences on another mediator of neural plasticity, BDNF responsivity, was a surprise in the current study. Given the impact of BDNF on the integrity of the intestinal mucosal barrier and evidence of microbiome dysbiosis in antibiotic-treated mice (Bistoletti et al., 2019; Li et al., 2018), more research is necessary to further elucidate the interactive roles of BDNF and the gut microbe ecosystem in chronic stress contexts.

In conclusion, the exploration of several dimensions of the stress response in the current study (i.e., endocrine, neural and behavioral dimensions) was employed to provide a perspective of the complex interactions among relevant variables associated with the stress response and the restoration of gastrointestinal dysbiosis. Due to practical challenges associated with space and resources, it was only feasible to conduct the present study with one sex (i.e., males). To increase translational value, however, it is important for future investigations to include both males and females since depression is more prevalent in human females (Albert, 2015; Myles et al., 2020a, 2020b; Yang et al., 2015). Further, clarification of the most effective ways to administer probiotics, as well as timing and dosage, are required to more fully understand the effects of these supplements (Myles et al., 2020a, 2020b). Tracking natural changes in the ecosystem of the microbiome following chronic unpredictable stress exposure in the absence of microbiota-targeted intervention will also contribute valuable findings to our understanding of stress-induced dysbiosis and allostatic load. Despite the limitations of a single study, these results add to an expanding knowledge base supporting familiar age-old maxims about gut-related mental states [e.g., idioms including gut-wrenching decisions, making gutsy responses, or experiencing butterflies in one's stomach (Foster et al., 2017)]. Thus, the vast gastrointestinal system appears to provide an important threat-detecting portal that has an influential impact on adaptive responses that maintain mental health.

## CRediT authorship contribution statement

**Nick Natale**: study conception and design, collection and analysis of data; manuscript preparation, **Molly Kent**: study conception and design, collection and analysis of data; manuscript preparation, **Nathan Fox**: data collection, **Dylan Vavra**: data collection, **Kelly Lambert**: study conception and design, collection and analysis of data; manuscript preparation.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibneur.2021.10.004.

## References


Abdel-Haq, R., Schlachetzki, J.C.M., Glass, C.K., Mazmanian, S.K., 2019. Microbiome–microglia connections via the gut–brain axis. J. Exp. Med. 216 (1), 41–59.

Albert, P.R., 2015. Why is depression more prevalent in women? J. Psychiatry Neurosci.: JPN 40 (4), 219–221.

Bardi, M., Hampton, J.E., Lambert, K.G., 2010. Fecal dehydroepiandrosterone (DHEA) immunoreactivity as a noninvasive index of circulating DHEA activity in young male laboratory rats. Comp. Med. 60 (6), 455–460.

Bardi, M., Rhone, A.P., Franssen, C.L., Hampton, J.E., Shea, E.A., Hyer, M.M., Huber, J., Lambert, K.G., 2012. Behavioral training and predisposed coping strategies interact to influence resilience in male Long-Evans rats: implications for depression. Stress 15 (3), 306–317.

Bellavance, M.-A., Rivest, S., 2014. The HPA–immune axis and the immunomodulatory actions of glucocorticoids in the brain. Front. Immunol. 5, 136.

Benton, T.D., Ifeagwu, J.A., Smith-Whitley, K., 2007a. Anxiety and depression in children and adolescents with sickle cell disease. Curr. Psychiatry Rep. 9 (2), 114–121. https://doi.org/10.1007/s11920-007-0080-0.

Benton, T., Staab, J., Evans, D.L., 2007b. Medical co-morbidity in depressive disorders. Ann. Clin. Psychiatry: Off. J. Am. Acad. Clin. Psychiatr. 19 (4), 289–303.

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J., Deng, Y., Blennerhassett, P., Macri, J., McCoy, K.D., Verdu, E.F., Collins, S.M., 2011. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141 (2), 599–609, 609.e1-3.

Bermúdez-Humarán, L.G., Salinas, E., Ortiz, G.G., Ramirez-Jirano, L.J., Morales, J.A., Bitzer-Quintero, O.K., 2019. From probiotics to psychobiotics: live beneficial bacteria which act on the brain-gut axis. Nutrients 11 (4). https://doi.org/10.3390/nu11040890.

Bistoletti, M., Caputi, V., Baranzini, N., Marchesi, N., Filpa, V., Marsilio, I., Cerantola, S., Terova, G., Baj, A., Grimaldi, A., Pascale, A., Frigo, G., Crema, F., Giron, M.C., Giaroni, C., 2019. Antibiotic treatment-induced dysbiosis differently affects BDNF and TrkB expression in the brain and in the gut of juvenile mice. PLoS One 14 (2), e0212856.

Boehme, M., Guzzetta, K.E., Bastiaanssen, T.F.S., et al., 2021. Microbiota from young mice counteracts selective age-associated behavioral deficits. Nat. Aging 1, 666–676. https://doi.org/10.1038/s43587-021-00093-9.

Bonaz, B., Bazin, T., Pellissier, S., 2018. The Vagus nerve at the interface of the microbiota-gut-brain axis. Front. Neurosci. 12, 49.

Bravo, J.A., Forsythe, P., Chew, M.V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J., Cryan, J.F., 2018. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc. Natl. Acad. Sci. U S A 108 (38), 16050–16055. https://doi.org/10.1073/pnas.1102999108. Epub 2011 Aug 29. PMID: 21876150; PMCID: PMC3179073.

Buffington, S.A., Di Prisco, G.V., Auchtung, T.A., Ajami, N.J., Petrosino, J.F., Costa-Mattioli, M., 2016. Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. Cell 165 (7), 1762–1775.

Calabrese, F., Rossetti, A.C., Racagni, G., Gass, P., Riva, M.A., Molteni, R., 2014. Brain-derived neurotrophic factor: a bridge between inflammation and neuroplasticity. Front. Cell. Neurosci. 8, 430.

Callaghan, B.L., Cowan, C.S., Richardson, R., 2016. Treating generational stress: effect of paternal stress on development of memory and extinction in offspring Is reversed by probiotic treatment. Psychol. Sci. 27 (9), 1171–1180.

Cani, P.D., Everard, A., 2016. Talking microbes: when gut bacteria interact with diet and host organs. Mol. Nutr. Food Res. 60 (1), 58–66.

Cani, P.D., Plovier, H., Van Hul, M., Geurts, L., Delzenne, N.M., Druart, C., Everard, A., 2016. Endocannabinoids–at the crossroads between the gut microbiota and host metabolism. Nat. Rev. Endocrinol. 12 (3), 133–143.

Capuco, A., Urits, I., Hasoon, J., Chun, R., Gerald, B., Wang, J.K., Kassem, H., Ngo, A.L., Abd-Elsayed, A., Simopoulos, T., Kaye, A.D., Viswanath, O., 2020. Current perspectives on gut microbiome dysbiosis and depression. Adv. Ther. 37 (4), 1328–1346. https://doi.org/10.1007/s12325-020-01272-7. Epub 2020 Mar 4. PMID: 32130662; PMCID: PMC7140737.

Cheng, L.-H., Liu, Y.-W., Wu, C.-C., Wang, S., Tsai, Y.-C., 2019. Psychobiotics in mental health, neurodegenerative and neurodevelopmental disorders. J. Food Drug Anal. 27 (3), 632–648.

Chunchai, T., Thunapong, W., Yasom, S., Wanchai, K., Eaimworawuthikul, S., Metzler, G., Lungkaphin, A., Pongchaidecha, A., Sirilun, S., Chaiyasut, C., Pratchayasakul, W., Thiennimitr, P., Chattipakorn, N., Chattipakorn, S.C., 2018. Decreased microglial activation through gut-brain axis by prebiotics, probiotics, or synbiotics effectively restored cognitive function in obese-insulin resistant rats. J. Neuroinflamm. 15 (1), 11.

Cowan, C.S., Callaghan, B.L., Richardson, R., 2016. The effects of a probiotic formulation (Lactobacillus rhamnosus and L. helveticus) on developmental trajectories of emotional learning in stressed infant rats. Transl. Psychiatry 6 (5), e823.

Cowan, C.S.M., Stylianakis, A.A., Richardson, R., 2019a. Early-life stress, microbiota, and brain development: probiotics reverse the effects of maternal separation on neural circuits underpinning fear expression and extinction in infant rats. Dev. Cogn. Neurosci. 37, 100627.

Cowan, C.S.M., Richardson, R., 2019b. Early-life stress leads to sex-dependent changes in pubertal timing in rats that are reversed by a probiotic formulation. Dev. Psychobiol. 61 (5), 679–687.

Crumeyrolle-Arias, M., Jaglin, M., Bruneau, A., Vancassel, S., Cardona, A., Daugé, V., Naudon, L., Rabot, S., 2014. Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. Psychoneuroendocrinology 42, 207–217.

Cryan, J.F., Dinan, T.G., 2012. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat. Rev. Neurosci. 13 (10), 701–712.

Daskalakis, N.P., De Kloet, E.R., Yehuda, R., Malaspina, D., Kranz, T.M., 2015. Early life stress effects on glucocorticoid—BDNF interplay in the hippocampus. Front. Mol. Neurosci. 8, 181.

Davis, M.T., Holmes, S.E., Pietrzak, R.H., Esterlis, I., 2017. Neurobiology of chronic stress-related psychiatric disorders: evidence from molecular imaging studies. Chronic Stress (Thousand Oaks, Calif.) 1. https://doi.org/10.1177/2470547017710916.

de Kloet, E.R., Molendijk, M.L., 2016. Coping with the forced swim stressor: towards understanding an adaptive mMechanism. Neural. Plasticity 2016, 6503162.

Dienes, K.A., Hazel, N.A., Hammen, C.L., 2013. Cortisol secretion in depressed, and at-risk adults. Psychoneuroendocrinology 38 (6), 927–940.

Dinan, T.G., Cryan, J.F., 2016. Mood by microbe: towards clinical translation. Genome Med. 8 (1) https://doi.org/10.1186/s13073-016-0292-1.

Dobbs, D., 2017. The smartphone psychiatrist. Atlantic 320, 78–86.

Drossman, D.A., 2006. The functional gastrointestinal disorders and the Rome III process. Gastroenterology 130 (5), 1377–1390.

Erny, D., Hrabě de Angelis, A.L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., Keren-Shaul, H., Mahlakoiv, T., Jakobshagen, K., Buch, T., Schwierzeck, V., Utermöhlen, O., Chun, E., Garrett, W.S., McCoy, K.D., Diefenbach, A., Staeheli, P., Stecher, B., Amit, I., Prinz, M., 2015. Host microbiota constantly control maturation and function of microglia in the CNS. Nat. Neurosci. 18 (7), 965–977.

Erny, D., Hrabě de Angelis, A.L., Prinz, M., 2017. Communicating systems in the body: how microbiota and microglia cooperate. Immunology 150 (1), 7–15.

Foster, J.A., McVey Neufeld, K.-A., 2013. Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci. 36 (5), 305–312.

Foster, J.A., Rinaman, L., Cryan, J.F., 2017. Stress & the gut-brain axis: regulation by the microbiome. Neurobiol. Stress 7, 124–136.

Frenois, F., Moreau, M., O'Connor, J., Lawson, M., Micon, C., Lestage, J., Kelley, K.W., Dantzer, R., Castanon, N., 2007. Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that parallel the expression of depressive-like behavior. Psychoneuroendocrinology 32 (5), 516–531.

Friedrich, M.J., 2017. Depression Is the leading cause of disability around the world. JAMA: J. Am. Med. Assoc. 317 (15), 1517.

Gareau, M.G., Jury, J., MacQueen, G., Sherman, P.M., Perdue, M.H., 2007. Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. Gut 56 (11), 1522–1528.

Geng, C., Guo, Y., Wang, C., Liao, D., Han, W., Zhang, J., Jiang, P., 2020. Systematic impacts of chronic unpredictable mild stress on metabolomics in rats. Sci. Rep. 10 (1) https://doi.org/10.1038/s41598-020-57566-x.

Guo, Y., Du, X., Bian, Y., Wang, S., 2020. Chronic unpredictable stress-induced reproductive deficits were prevented by probiotics. Reprod. Biol. 20 (2), 175–183. https://doi.org/10.1016/j.repbio.2020.03.005. Epub 2020 Apr 5. PMID: 32265160.

Hariri, A.R., Holmes, A., 2015. Finding translation in stress research. Nat. Neurosci. 18 (10), 1347–1352.

Peng, H.-H, Tsai, T.-C., Huang, W.-Y., Wu, H.-M., Hsu, K.-S., 2019. Probiotic treatment restores normal developmental trajectories of fear memory retention in maternally separated infant rats. Neuropharmacology 153, 53–62.

Hyman, S.E., 2012. Revolution stalled. Sci. Transl. Med. 4 (155), 155cm11.

Hyman, S.E., 2014. Revitalizing psychiatric therapeutics. Neuropsychopharmacology 39 (1), 220–229. https://doi.org/10.1038/npp.2013.181.

Insel, T.R., 2009. Disruptive insights in psychiatry: transforming a clinical discipline. J. Clin. Investig. 119 (4), 700–705.

Jin, Y., Sun, L.H., Yang, W., Cui, R.J., Xu, S.B., 2019. The role of BDNF in the neuroimmune axis regulation of mood disorders. Front. Neurol. 10, 515.

Johnson, K.V.-A., Foster, K.R., 2018. Why does the microbiome affect behaviour? Nat. Rev. Microbiol. 16 (10), 647–655.

Karl, J.P., Hatch, A.M., Arcidiacono, S.M., 2018. Effects of psychological, environmental and physical stressors on the gut microbiota (https://www.frontiersin.org/articles/). Frontiers. https://doi.org/10.3389/fmicb.2018.02013/full.

Kent, M., Bardi, M., Hazelgrove, A., Sewell, K., Kirk, E., Thompson, B., Trexler, K., Terhune-Cotter, B., Lambert, K., 2017. Profiling coping strategies in male and female rats: potential neurobehavioral markers of increased resilience to depressive symptoms. Hormones Behav. 95, 33–43.

Kent, M., Scott, S., Lambert, S., Kirk, E., Terhune-Cotter, B., Thompson, B., Neal, S., Dozier, B., Bardi, M., Lambert, K., 2018. Contingency training alters neurobiological components of emotional resilience in male and female rats. Neuroscience 386, 121–136.

Kho, Z.Y., Lal, S.K., 2018. The human gut microbiome–a potential controller of wellness and disease. Front. Microbiol. 9, 1835.

Koizumi, R., Kiyokawa, Y., Mikami, K., Ishii, A., Tanaka, K.D., Tanikawa, T., Takeuchi, Y., 2018. Structural differences in the brain between wild and laboratory rats (*Rattus norvegicus*): potential contribution to wariness. J. Vet. Med. Sc. 80, 1054–1060. https://doi.org/10.1292/jvms.18-0052.

Lambert, K.G., Hyer, M.M., Rzucidlo, A.A., Bergeron, T., Landis, T., Bardi, M., 2014. Contingency-based emotional resilience: effort-based reward training and flexible coping lead to adaptive responses to uncertainty in male rats. Front. Behav. Neurosci. 8, 124.

Lammert, C.R., Frost, E.L., Bolte, A.C., Paysour, M.J., Shaw, M.E., Bellinger, C.E., Weigel, T.K., Zunder, E.R., Lukens, J.R., 2018. Cutting edge: critical roles for microbiota-mediated regulation of the immune system in a prenatal immune activation model of autism. J. Immunol. 201 (3), 845–850. https://doi.org/10.4049/jimmunol.1701755.

Lalitsuradej, E., Sivamaruthi, B.S., Sirilun, S., Sittiprapaporn, P., Peerajan, S., Chaiyasut, C., 2020. The effect of supplementation of Lactobacillus paracasei HII01 on salivary cortisol, and dehydroepiandrosterone sulfate (DHEA-S) levels. Asian J. Med. Sci. 11 (1), 12–15. https://doi.org/10.3126/ajms.v11i1.26500.

Lee, B.-H., Kim, Y.-K., 2010. The roles of BDNF in the pathophysiology of major depression and in antidepressant treatment. Psychiatry Investig. 7 (4), 231–235.

Leung, K., Thuret, S., 2015. Gut microbiota: a modulator of brain plasticity and cognitive function in ageing. Healthcare 3, 898–916.

Li, C., Cai, Y.-Y., Yan, Z.-X., 2018. Brain-derived neurotrophic factor preserves intestinal mucosal barrier function and alters gut microbiota in mice. Kaohsiung J. Med. Sci. 34 (3), 134–141.

Liu, R.T., 2017. The microbiome as a novel paradigm in studying stress and mental health. Am. Psychol. 72 (7), 655–667.

Lovell, R.M., Ford, A.C., 2012. Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. Clin. Gastroenterol. Hepatol.: Offi. Clin. Pract. J. Am. Gastroenterol. Assoc. 10 (7), 712–721 e4.

Luczynski, P., Neufeld, K.-A.M., Oriach, C.S., Clarke, G., Dinan, T.G., Cryan, J.F., 2016. Growing up in a bubble: using germ-free animals to assess the influence of the gut microbiota on brain and behavior. Int. J. Neuropsychopharmacol. 19 (8), yw020. https://doi.org/10.1093/ijnp/pyw020.

Malick, M., Gilbert, K., Daniel, J., Arseneault-Breard, J., Tompkins, T.A., Godbout, R., Rousseau, G., 2015. Vagotomy prevents the effect of probiotics on caspase activity in a model of postmyocardial infarction depression. Neurogastroenterol Motil. 27 (5), 663–671. https://doi.org/10.1111/nmo.12540. Epub 2015 Mar 18. PMID: 25786501.

Manley, K., Han, W., Zelin, G., Lawrence, D.A., 2018. Crosstalk between the immune, endocrine, and nervous systems in immunotoxicology. Curr. Opin. Toxicol. 10, 37–45.

Marin, I.A., Goertz, J.E., Ren, T., Rich, S.S., Onengut-Gumuscu, S., Farber, E., Wu, M., Overall, C.C., Kipnis, J., Gaultier, A., 2017. Microbiota alteration is associated with the development of stress-induced despair behavior. Sci. Rep. 7, 43859.

McGinty, J.F., Whitfield Jr., T.W., Berglind, W.J., 2010. Brain-derived neurotrophic factor and cocaine addiction. Brain Res. 1314, 183–193.

Molendijk, M.L., de Kloet, E.R., 2015. Immobility in the forced swim test is adaptive and does not reflect depression. Psychoneuroendocrinology Vol. 62, 389–391. https://doi.org/10.1016/j.psyneuen.2015.08.028.

Morgan 3rd, C.A., Rasmusson, A., Pietrzak, R.H., Coric, V., Southwick, S.M., 2009. Relationships among plasma dehydroepiandrosterone and dehydroepiandrosterone sulfate, cortisol, symptoms of dissociation, and objective performance in humans exposed to underwater navigation stress. Biol. Psychiatry 66 (4), 334–340. https://doi.org/10.1016/j.biopsych.2009.04.004. Epub 2009 Jun 5. PMID: 19500775.

Moser, G., Fournier, C., Peter, J., 2018. Intestinal microbiome-gut-brain axis and irritable bowel syndrome. Wiener Medizinische Wochenschrift 168 (3–4), 62–66.

Munshi, S., Loh, M.K., Ferrara, N., DeJoseph, M.R., Ritger, A., Padival, M., Record, M.J., Urban, J.H., Rosenkranz, J.A., 2020. Repeated stress induces a pro-inflammatory state, increases amygdala neuronal and microglial activation, and causes anxiety in adult male rats. Brain Behav. Immun. 84, 180–199. https://doi.org/10.1016/j.bbi.2019.11.023.

Myles, E.M., O'Leary, M.E., Romkey, I.D., Piano, A., de Carvalho, V., Tompkins, T.A., Perrot, T.S., 2020a. Guidelines for best parctice in placebo-controlled experimental students on probiotics in rodent animal models. Benef. Microbes 11 (3), 245–254.

Myles, E.M., O'Leary, M.E., Smith, R., MacPherson, C.W., Oprea, A., Melanson, E.H., Tompkins, T.A., Perrot, T.S., 2020b. Supplementation with combined *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 across development reveals sex differences in physiological and behavioral effects of western diet in Long-Evans rats. Microorganisms 8, 1527.

Nikolova, Y.S., Misquitta, K.A., Rocco, B.R., Prevot, T.D., Knodt, A.R., Ellegood, J., Voineskos, A.N., Lerch, J.P., Hariri, A.R., Sibille, E., Banasr, M., 2018. Shifting priorities: highly conserved behavioral and brain network adaptations to chronic stress across species. Transl. Psychiatry 8 (1), 26. https://doi.org/10.1038/s41398-017-0083-5.

Papalini, S., Michels, F., Kohn, N., Wegman, J., van Hemert, S., Roelofs, K., Arias-Vasquez, A., & Aarts, E. (2018). Stress matters: a double-blind, randomized controlled trial on the effects of a multispecies probiotic on neurocognition. In bioRxiv (p. 263673). ⟨https://doi.org/10.1101/263673⟩.

Park, A.J., Bercik, P., Huang, X., Blennerhassett, P., Sinclair, D.D., Lu, J., Deng, Y., Bergonzelli, G., McLean, P., Collins, S.M., Verdu, E.F., 2011. The anxiolytic effect of bifidobacterium longum Ncc3001 requires vagal integrity for gut-brain communication. S – 18 Gastroenterology 140 (5). https://doi.org/10.1016/s0016-5085(11)60072-3.

Partrick, K.A., Chassaing, B., Beach, L.Q., McCann, K.E., Gewirtz, A.T., Huhman, K.L., 2018. Acute and repeated exposure to social stress reduces gut microbiota diversity in Syrian hamsters. Behav. Brain Res. 345, 39–48.

Perez-Burgos, A., Wang, B., Mao, Y.-K., Mistry, B., McVey Neufeld, K.-A., Bienenstock, J., Kunze, W., 2013. Psychoactive bacteria Lactobacillus rhamnosus (JB-1) elicits rapid frequency facilitation in vagal afferents. Am. J. 304 (2), G211–G220.

Poroyko, V.A., Carreras, A., Khalyfa, A., Khalyfa, A.A., Leone, V., Peris, E., Almendros, I., Gileles-Hillel, A., Qiao, Z., Hubert, N., Farré, R., Chang, E.B., Gozal, D., 2016. Chronic sleep disruption alters gut microbiota, induces systemic and adipose tissue inflammation and insulin resistance in mice. Sci. Rep. 6, 35405.

Rea, K., Dinan, T.G., Cryan, J.F., 2016. The microbiome: a key regulator of stress and neuroinflammation. Neurobiol. Stress 4, 23–33.

Redlich, R., Opel, N., Förster, K., Engelen, J., Dannlowski, U., 2018. Structural neuroimaging of maltreatment and inflammation in depression. Inflamm. Immun. Depress. 287–300. https://doi.org/10.1016/b978-0-12-811073-7.00016-7.

Sampson, T.R., Mazmanian, S.K., 2015. Control of brain development, function, and behavior by the microbiome. Cell host & microbe 17 (5), 565–576. https://doi.org/10.1016/j.chom.2015.04.011.

Sarkar, A., Lehto, S.M., Harty, S., Dinan, T.G., Cryan, J.F., Burnet, P.W.J., 2016. Psychobiotics and the manipulation of bacteria-gut-brain signals. Trends Neurosci. 39 (11), 763–781.

Scarola, S., Kent, M., Neal, S., Trejo, J.P., Bardi, M., Lambert, K., 2020. Postpartum environmental challenges alter maternal responsiveness and offspring development. Hormones Behav. 122, 104761.

Sharon, G., Cruz, N.J., Kang, D.-W., Gandal, M.J., Wang, B., Kim, Y.-M., Zink, E.M., Casey, C.P., Taylor, B.C., Lane, C.J., Bramer, L.M., Isern, N.G., Hoyt, D.W., Noecker, C., Sweredoski, M.J., Moradian, A., Borenstein, E., Jansson, J.K., Knight, R., Mazmanian, S.K., 2019. Human gut microbiota from autism spectrum disorder promote behavioral symptoms in mice. Cell 177 (6), 1600–1618 e17.

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.-N., Kubo, C., Koga, Y., 2004. Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. J. Physiol. 558 (1), 263–275.

Thion, M.S., Low, D., Silvin, A., Chen, J., Grisel, P., Schulte-Schrepping, J., Blecher, R., Ulas, T., Squarzoni, P., Hoeffel, G., Coulpier, F., Siopi, E., David, F.S., Scholz, C., Shihui, F., Lum, J., Amoyo, A.A., Larbi, A., Poidinger, M., Garel, S., 2018. Microbiome influences prenatal and adult microglia in a sex-specific manner. Cell 172 (3), 500–516 e16.

Vlasova, A.N., Kandasamy, S., Chattha, K.S., Rajashekara, G., Saif, L.J., 2016. Comparison of probiotic lactobacilli and bifidobacteria effects, immune responses and rotavirus vaccines and infection in different host species. Vet. Immunol. Immunopathol. 172, 72–84.

Vuong, H.E., Hsiao, E.Y., 2017. Emerging roles for the gut microbiome in autism spectrum disorder. Biol. Psychiatry 81 (5), 411–423.

Wang, H., Lee, I.-S., Braun, C., Enck, P., 2016. Effect of probiotics on central nervous system functions in animals and humans: a systematic review. J. Neurogastroenterol. Motility 22 (4), 589–605.

Wang, Y., Wang, Z., Wang, Y., Li, F., Jia, J., Song, X., Qin, S., Wang, R., Jin, F., Kitazato, K., Wang, Y., 2018. The gut-microglia connection: implications for central nervous system diseases. Front. Immunol. 9, 2325.

Wohleb, E.S., 2016. Neuron–microglia interactions in mental health disorders: "For Better, and For Worse.". Front. Immunol. 7 https://doi.org/10.3389/fimmu.2016.00544.

Yang, L., Zhao, Y., Wang, Y., Liu, L., Zhang, X., Li, B., Cui, R., 2015. The effects of psychological stress on depression. Curr. Neuropharmacol. 13 (4), 494–504. https://doi.org/10.2174/1570159x1304150831150507.

Yehuda, R., & Daskalakis, N.P. (2015). *Programming HPA-axis by early life experience: Mechanisms of stress susceptibility and adaptation*. Frontiers Media SA.

Yu, H., Chen, Z.-Y., 2011. The role of BDNF in depression on the basis of its location in the neural circuitry. Acta Pharmacol. Sin. 32 (1), 3–11.

Zobel, A.W., Nickel, T., Sonntag, A., Uhr, M., Holsboer, F., Ising, M., 2001. Cortisol response in the combined dexamethasone/CRH test as predictor of relapse in patients with remitted depression. a prospective study. Journal of Psychiatric Research 35 (2), 83–94.