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Maternal probiotic *Lactocaseibacillus rhamnosus* HN001 treatment alters postpartum anxiety, cortical monoamines and gut microbiome

Joseph S. Lonstein^{1*}, Taryn A. Meinhardt¹, Pavlina Pavlidi², Nikos Kokras^{2,3}, Christina Dalla³, Thierry D. Charlier⁴, & Jodi L. Pawluski⁴

¹Behavioral Neuroscience Program
Department of Psychology
Michigan State University
East Lansing, MI, 48824 USA

²Department of Pharmacology
Medical School
National and Kapodistrian University of Athens
Mikras Asias 75
11527, Goudi, Athens, Greece.

³First Department of Psychiatry, Medical School
National and Kapodistrian University of Athens

⁴Univ Rennes, Inserm, EHESP, Irset (Institut de Recherche en Santé, Environnement et Travail), F-35000, Rennes, France

*To whom correspondence should be addressed: lonstein@msu.edu

ABSTRACT

Peripartum mood and anxiety disorders (PMADs) affect 15-20% of peripartum women and are well known to disrupt infant caregiving. A recent study in humans reported that anxiety and depressive symptoms could be alleviated by peripartum treatment with the probiotic, *Lactocaseibacillus rhamnosus* HN001. The current study determined the effects of chronic *Lactocaseibacillus rhamnosus* HN001 (HN001) treatment on postpartum affective and caregiving behaviors in a laboratory rodent model. Female rats were given probiotic overnight in drinking water, or untreated water, from the first day of pregnancy through postpartum day 10. To determine whether the HN001 effects were influenced by a background of stress, half the females underwent chronic variable pregnancy stress and the other half remained undisturbed.

The results revealed that, even without pregnancy stress, HN001 reduced postpartum anxiety-related behavior, increased variability in behavioral fragmentation when dams interacted with pups, increased time away from pups, and decreased prefrontal cortex norepinephrine (NE), dopamine (DA) and serotonin (5-HT). Probiotic plus stress consistently reduced the latency to float in the forced swim test, increased DA and 5-HT turnovers in the prefrontal cortex, increased hippocampal NE, and reduced hypothalamic DA. Fecal microbe alpha and beta diversities were lower postpartum than prepartum, which was prevented by probiotic treatment and/or stress. Across the entire sample there was a negative relationship between dams' postpartum anxiety behavior and fecal *Bacteroides dorei*. This study reveals novel information about how *L. rhamnosus HN001* influences postpartum behavior and microbiota-gut-brain physiology in female laboratory rats, with implications for probiotic supplement use by pregnant and postpartum women.

Keywords: gut-brain axis, maternal behavior, microbiome, monoamines, pregnancy, probiotics, stress, maternal brain

Although peripartum mood and anxiety disorders (PMADs) affect up to 15-20% of the millions of pregnant and postpartum people around the world each year, effective treatments and preventative options remain sparse (Stewart and Vigod, 2019). Increased attention to PMADs is desperately needed because there are few times in a woman's life when the consequences of having mental illness are as high. At their extreme, PMADs can contribute to maternal mortality, but more commonly are associated with reduced breastfeeding, lower maternal emotional and behavioral sensitivity to the infant, poor bonding, negative infant temperament, altered infant neurodevelopment, as well as emotional and behavioral problems in the children when they are older (Slomian et al., 2019; Rogers et al., 2020). In addition to these negative outcomes, untreated PMADs are estimated to cost \$14.2 billion just in the United States within the first 5 years after affected women give birth (Stewart and Vigod, 2019).

A growing area of research on the biological basis of peripartum mental illness has focussed on the role of the microbiota-gut-brain axis. The gut microbiome is comprised of a vast collection of microorganisms that are strongly linked to health and disease (Codagnone et al., 2019). These microorganisms are capable of producing metabolites and neurotransmitters that allow communication between the gut and the brain, with consequences for neural function (Dinan and Cryan, 2017). Recent research demonstrates that psychosocial stress or psychiatric symptoms impact the maternal gut microbiome. For instance, higher perceived psychosocial stress measured within the first few months postpartum in women is associated with a concurrent reduction in fecal microbiota species diversity (Long et al., 2023). Consistent with that finding, women with and without postpartum depression differ in their fecal microbe communities and their depression scores are significantly correlated with the levels of a number of individual microbes (Zhou et al., 2020). Furthermore, high general anxiety or emotional distress during

pregnancy are associated with concurrent changes in expression of particular fecal microbes (Hechler et al., 2019; Penalver Bernabe et al., 2023) and past-year intimate partner violence (a very common stressor in pregnant women) is associated with family-level changes in fecal microbes measured at parturition (Naude et al., 2020). Important for translational research on this topic, chronic pregnancy stress also reduces microbial diversity and alters levels of specific microbes in the feces of female laboratory rodents (Jasarevic et al., 2017; Antonson et al., 2020; Pawluski et al., 2023; Wang et al., 2023). Given these stress- and mental-health related changes in the pregnant and postpartum gut microbiome, a number of meta-analyses have suggested that while more research is certainly needed, targeting the maternal gut microbiome may help alleviate stress and mental health symptoms during pregnancy and postpartum (Desai et al., 2021).

Targeting the gut microbiome to influence its composition and function is commonly done with widely available and relatively low-cost probiotic, prebiotic, or synbiotic supplements (Sorbara and Palmer, 2022) and a small percentage of pregnant women already use these supplements to improve their health and possibly that of their fetuses (Freeman et al., 2016; Rutten et al., 2016). Although research is limited, a recent meta-analysis concluded there is some promising evidence that probiotic supplements can reduce depressive- and anxiety-symptoms in human mothers (Desai et al., 2021). Probiotic supplements can contain a variety of microbial species, but a recent double-blind, placebo-controlled study involving 380 women (Slykerman et al., 2017) found small but statistically significant reductions in postpartum depressive and anxiety symptoms from daily ingestion of *Lacticaseibacillus rhamnosus* HN001 (HN001) starting in the second trimester and continuing through 6 months postpartum. However, another study of obese women reported that probiotic treatment with HN001 combined with

Bifidobacterium animalis ssp. *lactis* 420 had little impact on their postpartum depressive and anxiety symptoms, but this may have been due to very low baseline levels of mental health symptoms in this cohort of mothers (Hulkkonene et al 2021). In laboratory rodent models, male rats orally administered HN001 had reduced stress-induced depressive-like behaviors and altered basal levels of key brain neurotransmitters including serotonin, dopamine and norepinephrine (Huang et al., 2022). These studies collectively suggest that HN001 may be promising in treating PMADs in some populations of women and that its mode of action is by altering neurotransmitter systems essential for positive mental health.

To further investigate the effects of HN001 on the microbiota-gut-brain axis relevant to PMADs, we used a laboratory animal model involving a 2 x 2 experimental design that led to four groups of pregnant and parous laboratory female rats that were given: 1) Regular Water/No Pregnancy Stress, 2) Probiotic Water/No Pregnancy Stress 3) Regular Water/Pregnancy Stress, 4) Probiotic Water/Pregnancy Stress. We hypothesized that HN001 probiotic treatment during pregnancy would influence dams' postpartum affective behaviors, maternal caregiving, and brain monoamines. Based on the extant literatures, we also expected some significant correlative relationships specifically between the mothers' postpartum anxiety-related behavior and their frontal cortex monoamines and their gut microbiome.

Methods

Subjects

Subjects were adult (60-90 days old) female Long-Evans rats purchased from Envigo Laboratories (Indianapolis, IN) and housed upon arrival at Michigan State University in groups of 2 or 3 in clear polypropylene cages (48 cm × 28 cm x 16 cm). They were maintained on a

12:12 light/dark cycle (lights on at 0700 h) and room temperature was 22 ± 1 °C. Estrous cycles were monitored by daily assessment of vaginal cytology. Subjects were mated on a day of proestrus with an experienced Long-Evans male and pregnancy confirmed the next morning by the presence of sperm in the vaginal cytology. Subjects were singly housed immediately upon confirmation of insemination. Litters were culled within 24 hr of parturition to each contain 4 males and 4 females. Procedures were in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Michigan State University Institutional Animal Care & Use Committee.

Probiotic Treatment

Starting at 16:00 hr after detection of pregnancy and continuing every evening through postpartum day (PPD) 9 (see Figure 1 for experimental timeline), drinking water bottles were removed from the females' home cages and subjects were given a clean bottle in their home cage containing distilled water ($n = 19$) or given a bottle containing distilled water with 10^9 CFU of powdered *L. rhamnosus* HN001 (Fonterra Co-Operative Group Ltd, New Zealand) ($n = 20$). The *L. rhamnosus* HN001 powder was shipped frozen and stored at -20° C until and between its daily use. The concentration we used was based on similar studies providing HN001 or other probiotics in drinking water to laboratory rodents (Abildgaard et al., 2017; Kruger et al., 2009). The bottles remained overnight as the subjects' only source of fluid, were removed at 10:00 hr the next morning, and their regular (i.e., all untreated) water bottles were then returned again until that evening. The probiotic solution was prepared fresh daily. Overnight bottles were weighed before and after each evening to measure daily fluid intake. Subjects started with 30 ml of fluid in their overnight bottles (Abildgaard et al., 2017), which was increased or decreased

daily for each animal if needed based on their intake the previous evening. No animal drank >50 ml in a single evening.

Repeated Variable Stress

To determine if the expected probiotic treatment effects required a background of chronic stress, a repeated variable stress paradigm began on pregnancy day 7 and concluded in the afternoon of pregnancy day 20 for half of the regular-water females and half of the probiotic-treated females. Two of nine mild-moderate stressors were applied each day and consisted of: (1) restraint in a ventilated cylindrical Plexiglas™ restrainer for 1 hr, (2) exposure to a cold room (4°C) for 1 hr within the home cage, (3) overnight food deprivation, with chow removed overnight and replaced the next morning, (4) home cage tilt at 45° for 4 hr, (5) wet bedding for 4 hr achieved by scattering 250 mL of distilled water in the home cage followed by rehousing in a new cage with clean bedding, (6) 80 dB white noise while in the home cage for 1 hr, (7) tail pinch using a plastic clothespin placed at the base of the tail for 5 min while in the home cage, (8) continuous light overnight while in the home cage, followed by return to the main colony room with regular light cycles the next morning, (9) strobing light (1 flash/sec) in the home cage while in an otherwise dark testing room, during the light cycle for 1 hr.

Stressors were presented in a pseudorandomized manner that prevented any stressor from being presented on two consecutive days. After the repeated variable stress paradigm ended, subjects were left alone until parturition (designated as postpartum day 0, PPO). Because pup behavior itself is altered by gestational stress, and stressed pup behavior influences maternal care given by dam, litters were comingled at birth and each subject (regardless of group) received an equal number of pups delivered by stressed and unstressed dams. Dam and pre-culling litter

weights were taken within 24 hr after parturition.

Postpartum Behavior Testing

Maternal Caregiving: Beginning the day after parturition (PPD1), subjects were observed daily for maternal behavior for 30 min every morning between 0900-1000 hr and again in the afternoon between 1400-1500 hr on PPD1-7 (see Figure 1) (Grieb et al., 2021). Experimenters stood quietly in the colony room and nests were first scored on a scale of 0-3 (0 = no nest, 1 = poor nest, 2 = partial nest, 3 = complete nest with high walls) and dams' behaviors were then recorded by hand every 30 sec. Recorded behaviors included both maternal (licking, mouthing/carrying, and sniffing pups, nest building, actively hovering over the litter, quiescently nursing in any posture) and non-maternal (self-grooming, eating/drinking, resting away from the litter, exploring) behaviors. Dams' behavioral fragmentation during the undisturbed observations was determined by assigning one point each time a dam's behavior scored during a 30-sec epoch differed from the behavior that dam displayed during the previous 30-sec epoch (Ivy et al., 2008).

Elevated Plus Maze: After the undisturbed maternal caregiving behavior observations on the afternoon of PPD5, subjects were transported in their home cage (with their litter present) to a behavior testing room illuminated by a 100W light bulb. The elevated plus-maze was elevated 50 cm from the floor and made of black plastic with four arms emerging from a 10 X 10-cm center square. Arms were 10 cm wide by 50 cm long, two of which had 40-cm high walls and two of which had no walls. Approximate illumination was 28 lux on the open arms, and 2 lux on the closed arms. Each subject was removed from their home cage and placed in the central square of the maze facing an open arm and released for 5 minutes. The home cage with the litter

was brought to the adjacent room. A low-light-sensitive video camera suspended above the maze relayed images to a laptop in an adjacent room. Recorded behaviors were analyzed with Solomon Coder and included time spent in the open and closed arms, and the frequency of entries into each arm. After testing, dams were removed from the maze, returned to their home cage containing their pups, and carried back to the colony room.

Saccharin Preference: To assess possibly reduced interest in a typically rewarding stimulus (i.e., anhedonia; see Liu et al., 2018), on the morning of PPD8 subjects were given a 24-hr acclimation period involving access to two bottles, one containing regular water and another containing sweet but non-caloric 0.1% saccharin in their home cage. Bottle placement was counterbalanced. Each bottle was weighed, their positions swapped after 12 hours, then removed and weighed again after the 24 hours had elapsed. On PPD9 following the 24-hr habituation period, food and both bottles were removed from the subjects' home cage for 4 hr. After the four-hr deprivation, the dams' food was replaced, and they were again given access to a bottle filled with regular water and a bottle of 0.1% saccharin solution. The pups were also removed from the home cage during the 1-hr test and placed in an incubator set at nest temperature (34° C). This was done because we previously found that dams are highly distracted during the choice test if the pups are present and, instead of attending to the bottles and drinking from them, they spend almost all their time in the nest interacting with the pups (Grieb and Lonstein, unpublished data; also see Medina et al., 2021). After the 1-h test, the bottles were removed and reweighed to measure intake and the pups were then returned to the home cage.

Forced Swim: The forced swim test (FST) is well known for its original use as a test of pharmacological agents' anti-depressant activity in laboratory rodents (Porsolt et al., 1977), although interpreting animals' behavior this test is regularly up for debate. It was here used to

both indicate possibly anti-depressant effects of HN001 as well as assess females' coping strategy in the face of an inescapable stressor (Molendijk and de Kloet, 2022). As we have previously done (Grieb et al., 2021), on the afternoon of PPD8 subjects were moved from the animal facility to a nearby behavior testing room for a pre-swim test. Rats were placed into a 50 cm × 20 cm Plexiglas cylinder 40 cm full of 24 °C water so that the tail could not touch the bottom of the container. The pretest was recorded by a suspended camera for 15 min, after which the subjects were removed from the apparatus, towel dried, and returned to their home cage with their pups. The apparatus was washed and refilled with fresh water between each animal. On the afternoon of PPD9, at least one hour after saccharin preference testing, rats were again placed into the same Plexiglas™ cylinder filled with fresh water. The test was digitally recorded for 10 min, after which the subjects were removed from the apparatus, towel dried, and briefly returned to their home cage. The latencies to begin and total times spent swimming and showing immobility (i.e., floating in the water only making movements necessary to maintain the head above water) were later scored using behavior coding software (Solomon Coder). The percentage of time spent floating during the 10-min test was used as the measure of behavioral despair. Within five min after the conclusion of testing, dams were euthanized with CO₂, rapidly decapitated, brains extracted and placed on dry ice, then stored at -80°C (Kokras et al., 2018). We obtained brains right after testing in order to assess dams' monoamine levels as contemporaneous as possible to their behavior, rather than hours later when the recent swim stress was more likely to acutely or permanently affect neurochemistry (e.g., Roth et al., 1982; Sudo et al., 1983).

High Performance Liquid Chromatography

Brains were removed from -80°C and the prefrontal cortex (including the anterior cingulate, prelimbic and infralimbic subregions), the entire hypothalamus, and the entire hippocampus were manually dissected out and placed on ice. Tissue samples were then homogenized and deproteinized in 500-800 μL of 0.2 N perchloric acid solution (Applichem, Darmstadt, Germany) containing 7.9 mM $\text{Na}_2\text{S}_2\text{O}_5$ and 1.3 mM Na_2EDTA (both from Riedel-de Haën AG, Seelze, Germany) and then centrifuged at 20,000g for 45 minutes at 4°C . The supernatant was collected and stored at -80°C until analysis while the pellets were further used for the measurement of protein concentration.

Analytical measurements were performed using an LKB2248 (*Pharmacia* LKB Biotechnology, Uppsala, *Sweden*) HPLC pump coupled with a BAS LC4C (Bioanalytical Systems, West Lafayette, IN, USA) electrochemical detector as previously described (Bessinis, Dalla et al. 2013), with some minor modifications. The working electrode was glassy carbon, the reference electrode was Ag/AgCl and the column used was Aquasil C18, 150mm X 2.1 mm, $5\mu\text{m}$ (Thermo Fisher Scientific, Massachusetts, USA). The voltage of the working electrode was set at +800mV. In all samples reverse phase ion pair chromatography was used to assay norepinephrine (NE), serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic (5-HIAA), as well as dopamine (DA) and its metabolites 3,4 dihydroxyphenylacetate (DOPAC), homovanillic acid (HVA) and 3-Methoxytyramine (3-MT). Because the methods were optimized for 5-HT and 5-HIAA analysis, the NE metabolites MHPG and VMA were not able to be measured. The mobile phase consisted of a 50 mM phosphate buffer regulated at pH 3.0, containing 300 mg/L octyl sulfate sodium salt (MP Biomedicals LLC, Irvine, CA, USA) as the ion pair agent and 20 mg/L Na_2EDTA ; acetonitrile (Merck, Darmstadt, Germany) was added at 8-10% v/v concentration.

The sensitivity of the assay was tested for each series of samples using external standards which were prepared daily in 0.2 N perchloric acid solution containing 7.9 mM Na₂S₂O₅ and 1.3mM Na₂EDTA. Samples were quantified by comparison of the area under the curve (AUC) against these reference external standards using a PC compatible HPLC software (Clarity, Data-Apex, Czech Republic). The 5-HT and DA turnover rates (5-HIAA/5-HT, HVA/DA, DOPAC/DA), were calculated as indices of serotonergic and dopaminergic activity, respectively. These indices have been repeatedly shown to correspond to serotonergic and dopaminergic neurotransmission activity, reflecting 5-HT release and/or metabolic activity.

Feces Collection and Microbiome Analysis:

All materials involved in feces collection were sterilized with 70% ethanol and allowed to dry. On pregnancy day 20 and postpartum day 10, each rat was removed from their home cage and placed in a clean polypropylene cage containing no bedding, food or water. An experimenter wearing clean nitrile gloves as well as single-use disposable face masks and laboratory coats checked the cages every 5 min until the rats excreted a fecal pellet. Most animals provided a pellet within the first 10 min. After a fecal pellet was discovered in the collection cage, rats were returned to their home cage. Experimenters changed their nitrile gloves and the fecal pellets were collected with sterilized forceps and placed in a mini-centrifuge tube. If the sterilized forceps touched anything other than the fecal pellet while attempting to retrieve it, a different sterilized forceps was used to collect the sample. Samples were stored at -80° C until processing.

Fecal samples were later thawed within their centrifuge tubes and ~100 mg of each sample taken from the interior of the pellets with spatulas sterilized between samples and samples sent to the Wayne State University Genomic Science Core (Detroit, MI) for full-length 16S rRNA gene sequencing. DNA was extracted from fecal samples using the Qiagen

PowerFecal Pro DNA kit (Qiagen) according to manufacturer's guidelines. Extracted DNA was quantified using the Qubit dsDNA High-Sensitivity Assay kit (Thermo-Fisher Invitrogen) and normalized to 5 ng/ μ l. The V3-V4 variable region of the 16S rRNA gene was amplified using 341F and 805R primer pair by following the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina), but with an increased input of 25 ng template DNA in a 50 μ l-volume reaction for initial PCR, then cleaned and indexed using Nextera XT v2 indices as described. Final indexed libraries were assessed on a 1.5% agarose gel and quantified using the Qubit dsDNA High-Sensitivity Assay kit (Thermo-Fisher Invitrogen). Cleaned libraries were pooled equally, and quality control of the final pool carried out on an Agilent Bioanalyzer 2100 using the High Sensitivity DNA chip assay (Agilent) and quantified by qPCR using the KAPA Library Quantification kit for Illumina (Roche KAPA). Sequencing was performed on a MiSeq using a 2 x 300bp v3 kit.

The quality of raw reads was checked using FastQC v0.11.5 followed by quality filtering using Trimmomatic v0.39 with the following parameters: SLIDINGWINDOW:5:20, MINLEN:200 AVGQUAL:20. A big data pipeline was used to infer Ribosomal Sequence Variants (RSVs) using the DADA2 v1.20 considering the following parameter: truncLen=c(280,210), trimLeft=c(17,21), maxEE=c(2,2), truncQ=c(2,2), maxN=0, rm.phix=TRUE. In brief, stringent quality filtering was performed with the FilterAndTrim function. Reads that mapped to the phiX genome were removed, forward (280 bp) and reverse (210 bp) reads were truncated to specific position, reads that contained unambiguous base or bases with quality score <2 were discarded, and the maximum number of expected errors per read was set to 2. Following that, DADA2 error correction and chimera removal steps were carried out to infer RSVs. Resulting non-chimeric RSVs were again chimera filtered using

reference-based chimera filtering implemented in USEARCH v11. Taxonomy was assigned to non-chimeric sequences using the assignTaxonomy function with the SILVA 138 reference database. Additionally, we used SPINGO for species-level classification whenever possible.

Data Analyses

Overnight fluid intake and maternal caregiving behaviors were analyzed with repeated-measures ANOVAs, with day as the within-subjects factor and pregnancy stress and probiotic treatment as the between-subjects factors, all with Greenhouse-Geisser correction. Because pregnancy-stressed females drank less fluid during most of the time of stress application (see Supplemental Results), subjects' total overnight fluid intake was collapsed across day (P1 through PP7) and used as a covariate in the data analyses.

Litter and dam weights measured soon after parturition, elevated plus maze, forced swim, saccharin preference, and brain data were analyzed with 2 (stress/no stress) x 2 (probiotic/no probiotic) ANOVAs. Grubb's tests were used on each dataset to detect single outliers, which when found were removed. Statistical significance for these analyses was indicated by $p < 0.05$. Partial eta squared (η^2) is reported to indicate effect sizes from each statistically significant ANOVA result with $\eta^2 = 0.01$ indicating small effects, $\eta^2 = 0.06$ indicating medium effects, and $\eta^2 \geq 0.14$ indicating large effects. Pearson correlations were used to determine linear relationships between postpartum elevated plus maze open-arm data with some of the brain monoamine and microbiome data. These anxiety data were specifically chosen for correlation analysis because they revealed the largest effect sizes (see below) and avoided statistical misinterpretation involved in correlating every behavioral variable measure in this study with every biological variable.

For alpha diversity analysis, Wilcoxon tests were used to determine statistical significance in the between-group and within-group analysis. Paired testing was performed whenever possible. For beta diversity analysis, compositionally coherent Aitchison distance method and applied pairwise Permutational Multivariate Analysis of Variance (PERMANOVA) were used to quantify either community-level differences between groups or timepoints using 9999 permutations. This test evaluates if group or timepoint have any significant effect on the overall gut microbiota composition. To ensure the reliability of the results, we also evaluated the assumption of homogeneity of variance (i.e., equal spread for groups) by betadisper analysis. Whenever possible, we evaluated PC1 and PC2 projections for group-wise or timepoint-wise differences using paired or unpaired Wilcoxon tests.

Taxa that varied in abundance between groups and timepoints were identified using ALDEx2 (ANOVA-Like Differential Expression), a compositionally-robust differential abundance analysis approach. ALDEx2 estimates per-feature technical variation within each sample using Monte-Carlo instances ($n = 512$) drawn from the Dirichlet distribution (doi: 10.1186/2049-2618-2-15). This distribution maintains the proportional nature of the data (doi: 10.3389/fmicb.2017.02224). ALDEx2 uses the Centred Log-Ratio (CLR) transformation (normalisation) that ensures the data are scale invariant and sub-compositionally coherent. ALDEx2 measures the effect sizes and returns p-values as calculated by Wilcoxon tests along with Benjamini-Hochberg (BH) correction due to multiple testing. Effect sizes were determined by the ratio of the between-group differences to the maximum of within-group differences so, by default, ALDEx2 developers recommend effect-size cut-off of >1 . For this analysis, we filtered out rare and low abundant taxa (recommended to decrease sparsity in the dataset, and our default

and tested approach) by retaining only those taxa is present in >5% of the sample with mean abundance of >0.01% in at least one of the two groups being compared.

Delta change (change between the pregnant and postpartum timepoints) was then calculated between groups. For this analysis, raw abundance counts were CLR normalized before calculating delta change and then statistical analyses were performed using paired Wilcoxon test followed by multiple testing correction with Benjamini-Hochberg (BH). Effect size differences for each taxa were calculated using the ALDEx2 equivalent effect size measurement method using `distE.effect` function from the `distEffect` R based package. For correlation analysis between microbes and behavior, CLR abundance of each taxa were correlated with subjects' percentage of frequency and duration in the open arms of the elevated plus-maze using Spearman correlation tests using `cor.test` function in R followed by multiple testing correction using the Benjamini-Hochberg (BH) method. We used non-parametric Spearman correlation as it can better handle outliers and is more suited for microbiota data which often have non-normal distribution.

Results

Dams' Fluid Intake

As expected, females drank more fluid overnight as pregnancy and lactation progressed ($F(7,245) = 10.50, p < 0.001, \eta^2_p = 0.23$). There was no interaction between treatment day and fluid type (i.e., regular water or probiotic-treated water) on overnight intake ($F(7,245) = 0.46, p = 0.87$), but there was a significant interaction between treatment day and stress ($F(7,245) = 3.17, p < 0.001, \eta^2_p = 0.08$), with pregnancy-stressed dams drinking somewhat less than unstressed dams starting a few days after the stressors began (Supplemental Figure 1). The 3-way interaction among day, fluid type, and stress on overnight intake was not significant ($F(7,245) = 1.21, p =$

0.30).

Elevated Plus Maze

There were significant main effects of probiotic ($F(1,37) = 4.58, p = 0.04, \eta^2_p = 0.13$) and pregnancy stress ($F(1,37) = 10.81, p = 0.002, \eta^2_p = 0.25$) on the percentage of entries that dams made into the open arms (Figure 2A), unexpectedly indicating less anxiety as a consequence of either treatment. The interaction between the factors was not significant ($F(1,37) = 1.66, p = 0.21$). The percentage of time spent in the open arms was unaffected by treatment ($ps > 0.23$, Figure 2B), as was the frequencies of closed-arm entries ($ps > 0.16$) that latter indicating no treatment effects on general locomotion.

Saccharin Acclimation and Preference

Total amount of fluid intake (i.e., from both bottles), the percentage of intake that was 0.1% saccharine, and the percentage of intake that was regular water during the 24-hr acclimation period did not differ among groups ($ps > 0.19$; data not shown). During the 1-hr preference test, total intake was significantly lower in the stressed dams compared to unstressed dams ($F(1,35) = 4.42, p = 0.04, \eta^2_p = 0.13$), and more importantly so was their percentage of total intake that was from the bottle containing 0.1% saccharin ($F(1,35) = 5.93, p = 0.02; \eta^2_p = 0.18$) (Figure 2C), suggesting higher anhedonic state after pregnancy stress.

Forced Swim Test

There was a statistically significant main effect of probiotic ($F(1,32) = 4.82, p = 0.04; \eta^2_p = 0.13$), and an interaction between probiotic and stress ($F(1,32) = 6.09, p = 0.02, \eta^2_p = 0.16$), on

the latency to begin floating. Dams receiving both pregnancy stress and probiotic were considerably faster to float compared to dams in other groups (Figure 2D). The percentage of time spent floating was unaffected by stress ($F(1,32) = 3.32, p = 0.08$), probiotic ($F(1,32) = 0.03, p = 0.86$), or their interaction ($F(1,32) = 0.05, p = 0.83$) (Figure 2E).

Maternal Caregiving

Neither pregnancy stress ($F(1,34) = 3.60, p = 0.07$), probiotic treatment ($F(1,34) = 0.13, p = 0.72$), nor their interaction ($F(1,34) = 1.10, p = 0.03$) affected dam body weights measured soon after parturition. Similarly, pregnancy stress ($F(1,34) = 1.24, p = 0.27$), probiotic treatment ($F(1,34) = 0.45, p = .51$), nor their interaction ($F(1,34) = 0.01, p = 0.94$) affected litter weights measured soon after birth. Litter and dam body weights at sacrifice on PPD 10 were also unaffected by the two treatments or their interaction ($ps > 0.51$; data not shown).

There was a significant main effect of test day on the morning ratings of nest quality, with nest scores higher after PPD1 ($F(3.8, 129.6) = 2.49, p = 0.05, \eta^2_p = 0.07$). Despite all mothers building good nests each day (Supplemental Table 1), there was a significant main effect of stress ($F(1,34) = 4.67, p = 0.38, \eta^2_p = 0.12$) with stressed dams building slightly better nests than unstressed dams. There was no statistically significant day by probiotic interaction ($F(3.8, 129.6) = 2.39, p = 0.06$), day by stress interaction ($F(3.8, 129.6) = 0.51, p = 0.07$), or a three-way interaction ($F(3.8, 129.6) = 1.96, p = 0.11$) on morning nest scores.

Maternal behavior fragmentation during the daily undisturbed observations showed no statistically significant effect of day ($F(5.1, 174.5) = 2.20, p = 0.06$), no day by probiotic interaction ($F(5.1, 174.5) = 1.83, p = 0.11$), nor a day by stress interaction ($F(5.1, 174.5) = 0.95, p = 0.45$). There were also no main effects of probiotic or stress, nor an interaction between

them, on behavior fragmentation ($ps > 0.30$). There was, however, a three-way interaction ($F(5.1, 174.5) = 3.06, p = 0.01, \eta^2_p = 0.08$) among the factors. This likely was due to the relatively stable behavioral fragmentation that can be seen in the control group across days, while all three treatment groups showed notable variability in their fragmentation across days (Figure 3A). To better understand this interaction, each subject's lowest daily fragmentation score (i.e., their trough) was subtracted from their peak daily fragmentation score. Probiotic-treated dams had a significantly larger peak-to-trough difference, indicating greater change in fragmentation across test days ($F(1,34) = 5.80, p = 0.02, \eta^2_p = 0.15$) (Figure 3B).

There were numerous expected significant main effects of test day on dams' undisturbed behaviors in the home cage with pups (not shown), but only a few statistically significant treatment effects (Supplemental Table 1). There was an interaction between water type and stress on dams' inactivity away from the pups ($F(1,34) = 5.80, p = 0.02, \eta^2_p = 0.15$), due to dams singularly treated with stress or with probiotic showing less inactivity away from pups than the other two groups (particularly the regular water/no stress group; Figure 3C). There was also a significant 3-way day by water by stress interaction on dams' self-grooming ($F(5.5, 204) = 3.43, p = 0.004, \eta^2_p = 0.09$) due to a drop in self-grooming in the untreated controls particularly on PPD4 (Figure 3D).

Forebrain Monoamines

In the prefrontal cortex, probiotic treatment significantly reduced NE ($F(1,31) = 6.02, p = 0.02; \eta^2_p = 0.16$), DA ($F(1,31) = 7.68, p = 0.009; \eta^2_p = 0.20$) and 5-HT levels ($F(1,31) = 6.66, p = 0.015; \eta^2_p = 0.18$) (Figure 4A-C). Stress alone affected none of the monoamine measures, but there were significant probiotic by stress interactions on NE and DA with Probiotic/Stress dams

lower than the regular water/stress group. There were also probiotic by stress interactions on dopamine (HVA/DA) and serotonin (5-HIAA/5-HT) turnover ratios (Supplemental Table 2), in both cases with the Probiotic/Stress dams having the highest turnover.

In the hypothalamus, there was an interaction between probiotic and stress on DA levels, with the Probiotic/Stress group having lower DA levels than the Probiotic/No Stress group ($F(1,34) = 4.17, p = 0.05, \eta^2_p = 0.11$) (Supplemental Table 2). In the hippocampus, there was an interaction between probiotic and stress on NE levels, with the probiotic/stress group having higher NE levels than the Regular water/No Stress control group ($F(1,33) = 4.41, p = 0.04; \eta^2_p = 0.12$) (Supplemental Table 2).

Correlations between anxiety behavior and forebrain monoamines

The percentage of entries dams made into the open arms of the elevated plus maze was negatively correlated with NE levels in the hypothalamus ($r = -0.33, p = 0.05$, Figure 4D). The percentage of time mothers spent in the open arms was significantly negatively correlated with prefrontal cortex levels of DA ($r = -0.41, p = 0.02$) and 5-HT ($r = -0.37, p = 0.03$), but significantly positively correlated with DA turnover ($r = 0.35, p = 0.05$) (Figure 4E-G). No significant correlations were found between open-arm behavior in the EPM and hippocampal monoamines (all $r_s < 0.32, p_s > 0.24$).

Fecal Microbiota

Exploration of population-level changes from pregnancy to postpartum using beta diversity analysis suggested a strong effect of reproductive state (pregnant or postpartum) on microbiota composition, with a visible shift from pregnancy to postpartum across the PC1 axis,

indicating higher beta diversity postpartum (Figure 5A). Distance-based pairwise PERMANOVA analysis (Supplemental Table 3) and the principle projection-based Wilcoxon test indicated a significant difference from pregnancy to postpartum only for the Regular Water/No Stress (pseudoF = 1.31, $p = 0.05$) and Regular Water/Pregnancy Stress (pseudoF = 1.45, $p = 0.02$) groups. The groups did not differ from each other in their absolute change (postpartum - pregnancy) in alpha and beta diversity. The change in within-subject beta diversity across time was compared among the four groups, but there were no significant differences in the beta diversity changes, although all groups had changed significantly since pregnancy.

Alpha diversity was evaluated to compare between-group and within-group differences. Comparison of species diversity and richness revealed significant decreases in observed species index ($p = 0.015$) with a trend toward statistical significance detected for Shannon index ($p = 0.078$) between pregnancy and postpartum only for the Regular Water/No Stress group (Figure 5B). However, levels of time-related change in alpha diversity indices did not significantly differ between the groups.

Stack bar charts at the phylum-, family-, and genus-level taxa (Figure 6A) were generated for each group during pregnancy and postpartum to visualize differences in microbiota community structures between groups and across time. The top five phyla had >0.5% mean abundance across the samples and accounted for 99.5% of total bacterial sequences. The 16 family-level taxa had >0.5% mean abundance across the samples and accounted for 80.6% of total bacterial sequences (Figure 6B). Lastly, the 24 genus-level taxa had >0.5% mean abundance across the samples and accounted for 55.3% of total bacterial sequences (Figure 6C). After correcting the p -values for the many multiple comparisons, there were no statistically significant differences in individual taxa, family, genus or species either within groups when

comparing their pregnancy and postpartum fecal samples, or between groups when comparing the probiotic or stress treatments. That is, no particular microbial taxa, family, genus, or species were responsible for the reproductive timepoint or group differences in beta and alpha diversity reported above. Nonetheless, the most abundant microbe in the prepartum and postpartum fecal samples from our HN001-treated dams was indeed *Lacticaseibacillus*, being many times higher than any other microbe detected in their feces, and much higher than the *Lacticaseibacillus* levels found in the prepartum or postpartum feces of regular-water treated groups (Supplemental Figure 2).

Correlations Between Anxiety Behavior and Fecal Microbiota

There was a significant negative correlation between dams' percentage of time spent in the open arms of the elevated plus maze and their postpartum fecal levels of only *Bacteroides dorei* ($r = -0.58$, $q = 0.016$) (Figure 6D), after correcting for multiple comparisons.

Discussion

The possibility of ameliorating mental health disorders by targeting the microbiome-gut-brain axis has recently received tremendous attention (Sorbara and Palmer, 2022). Although most of the research using probiotic supplements to target the gut microbiome during pregnancy has focused on physical health outcomes of women or their infants (Gomez Arango et al., 2015; Dunlop et al., 2015), a few studies have investigated maternal mental health. As discussed above, the largest such study by Slykerman et al. (2017) was a randomized trial of >400 women who orally received *L. rhamnosis* HN001 (HN001) or placebo daily from 14-16 weeks of pregnancy through six months postpartum. When measured at the end of the study, women receiving

HN001 had significantly lower scores on the Edinburgh Postnatal Depression Scale (EPDS) and State-Trait Anxiety Inventory (STAI) compared to controls.

The current study administering HN001 during pregnancy and postpartum in laboratory rats was based on those promising results in women, as well as the ability of HN001 to prevent stress-induced depressive-like behaviors and alter central monoamines in male laboratory rats (Huang et al., 2022). Most notably, we found that chronic pregnancy-through-postpartum HN001 administration via drinking water was associated with lower anxiety-related behavior in an elevated plus maze, increased peak-to-trough variability in mothers' behavioral fragmentation while interacting with pups, reduced PFC monoamine levels (5-HT, DA, NE), and prevented the pregnancy-to-postpartum shift in gut microbiome beta diversity. Furthermore, HN001 treatment interacted with pregnancy stress to hasten adaptive coping in response to inescapable stress (i.e., forced swim), was associated with increased frequency that mothers spent away from their pups, and higher PFC 5-HT and DA turnover. Either alone or with pregnancy stress, HN001 also prevented the pregnancy-to-postpartum decrease in microbiome alpha diversity. Lastly, collapsed across groups, anxiety-related behavior was inversely correlated with postpartum fecal *Bacteroides dorei*.

Our finding that HN001 decreased anxiety-like behavior was expected based on the reduced anxiety in parous women treated with HN001 found by Slykerman et al. (2017) and consistent with studies showing that chronic treatment with other *Lactocaseibacillus* species or subspecies reduces anxiety in stressed parous rats tested at weaning (Yang et al., 2022) or in stressed or unstressed virgin female and male laboratory rodents (Lalonde and Strazielle, 2022). A few studies have instead found no anxiolytic effects of HN001 or other *Lactocaseibacillus* species in parous (Cowan et al., 2016) or non-parous rodents (Huang et al., 2022; Ma et al.,

2023). Apparent discrepancies may be due to differences in subject sex, reproductive experience, probiotic species given, or other factors such as employing stressful oral gavage to deliver probiotics. The postpartum gut microbiome is known to differ in diversity and/or microbial profile from that of pregnancy and potentially non-parous females or males (present results; Pawluski et al 2023), so it may uniquely respond when targeted with probiotics. In support, subsequent clinical studies by Slykerman and colleagues reported that the ability of HN001 to reduce mental health symptoms in their mild-to-moderately symptomatic parous women (Slykerman et al., 2017) was not found in a sample of highly anxious male or female nurses or in a sample of very low-stress pre-diabetic patients (Slykerman et al., 2022; Barthow et al., 2022). It may be possible that HN001 is the most effective at moderate levels of distress.

Interestingly, we also found that pregnancy stress was associated with less saccharin intake (a reflection of low hedonic pleasure; Lui et al., 2018). The magnitude of this effect may have been influenced by the rats undergoing the forced swim pre-exposure the day before the saccharin preference testing, such that the pregnancy-stressed dams may have been even more affected by the recent acute swim stress compared to the pregnancy unstressed controls in their saccharin intake. Indeed, a recent study in adult male rats reported that a recent acute stress decreased saccharin preference, but only in animals also subjected to a week of chronic variable stress that concluded a week before preference testing (Zurita et al., 2020). In any case, our results demonstrate that this anhedonic effect of pregnancy stress was not prevented by concomitant HN001 treatment. Unfortunately, there are no other studies using HN001 in pregnant and postpartum laboratory rodents to compare our saccharin preference test results with, but Slykerman et al. (2017) found that HN001 reduced both postpartum anxiety and depressive symptoms in women, making it very plausible that laboratory rats and women differ

in how chronic HN001 can temper their postpartum depression-like symptoms and behavior.

Similar to HN001, chronic pregnancy stress alone reduced postpartum anxiety-related behavior in the elevated-plus maze (i.e., increased percentage of entries made into open-arms). A very similar reduction in anxiety in stressed female rats tested at the end of pregnancy was recently found (Pawluski et al., 2023) and another study recently found no effect of chronic variable pregnancy stress on postpartum anxiety in rats (Vitale et al., in preparation). Such null effects of pregnancy stress on postpartum anxiety have been found before (Neumann et al., 2005; Pawluski et al., 2012; Leuner et al., 2014). This is in contrast to the typical expectation that repeated pregnancy stress increases postpartum anxiety in female rodents. Differences in details of the pregnancy stress (duration, frequency, type of stressors), the anxiety paradigms used (elevated plus maze, light/dark box, open field), conditions of testing (light or dark photophase) and strain and age of the animals may contribute to variability among studies. The ability of pregnancy stress, as well as HN001, to reduce anxiety-related behavior should be interpreted with caution until it can be tested in other rodent anxiety paradigms (e.g., light-dark box, zero maze). In addition, despite non-significant probiotic by stress interactions, these anxiety results should be interpreted in light of the apparent bimodal distribution in the percentage of open arm entries seen in the no-probiotic/no stress control mothers (Figure 2A).

Postpartum anxiety-related behavior across our entire sample of rats was inversely correlated with the dams' fecal *Bacteroides dorei*. No previous studies have linked this particular microbe with anxiety in any animal, but *B. dorei* and some other members of *Bacteroides* can produce the inhibitory neurotransmitter GABA within the gut (Yunes et al., 2016; Strandwitz et al., 2019) and lower levels of gut *Bacteroides* are associated with lower GABA in the mouse PFC (Hassan et al., 2019). Our negative correlation between *B. dorei* and anxiety-related

behavior also supports research showing that gut *Bacteroides* is lower in humans with generalized anxiety disorder compared with healthy controls (Jiang et al., 2018) and that antidepressant use (which presumably decreases depression and possibly anxiety) is associated with higher gut *B. dorei* (Zhernakova et al., 2016).

The forced-swim test is often used to assess rodents' coping strategy in the face of an inescapable stressor (Molendijk and de Kloet, 2022). Studies in stressed, nulliparous female and male rodents find that *Lactocaseibacillus* treatment reduces floating and increases struggling/swimming, interpreted by some as indicating less "depressive-like behaviors" (Mindus et al., 2021). Our mothers receiving both pregnancy stress and probiotic were instead considerably faster to begin floating compared to mothers in the other groups, possibly suggesting anti-depressant actions of the HN001. Increased duration of time spent floating in this test by postpartum mice given a mixed probiotic from mating through lactation was also recently reported (Figure S11 in Radford-Smith et al., 2022). It has instead been proposed that, rather than indicating changes in depressive-like behavior in response to a drug treatment, a faster latency or greater duration of floating in the forced swim test could also reflect an adaptive strategy to conserve energy in the face of a known, inescapable stressor. This would be particularly advantageous when energy requirements are tremendously taxed by pregnancy or lactation. This possibility is tempered by the fact that unmanipulated cycling and lactating rats, despite differences in energy balance, generally do not differ in forced-swim test behavior (see Lonstein et al., 2014). Instead, HN001 may help stressed mothers better remember the inescapable aspect of the test. All subjects were pre-exposed to the paradigm the day before testing, and would presumably remember such a stressful recent event. The almost immediate latency to float in the pregnancy stressed plus probiotic group could reflect better memory

formation, retention or retrieval. Indeed, administering other strains of *Lactocaseibacillus* can enhance various types of learning in rats and mice (Alemohammad et al., 2022) as well as in humans (Tooley, 2020). Importantly, while our treated animals had a considerably faster latency to begin floating, they did not differ from the other groups in their total duration of floating across the entire test, consistent with some other recent studies that report both floating variables (e.g., Stanic et al., 2021; Saavedra et al., 2021), and suggesting that the animals did not persist in employing this seemingly useful strategy.

HN001-treated females showed some small differences from control dams in their maternal caregiving. Specifically, HN001 significantly increased dams' across-time range in behavioral fragmentation while interacting with pups, and when combined with pregnancy stress HN001 increased frequency away from the pups; both effects are indicators of reduced consistency and quality of maternal caregiving. The only other studies examining probiotic effects on maternal care in rodents found no effects of postpartum *Lactobacillus rhamnosus* R0011 plus *L. helveticus* strain R0052 on dams' retrieval of pups (Cowan et al., 2016), and no effects of a mixture of 14 probiotics given from mating through weaning on pup retrieval or undisturbed maternal caregiving (Radford-Smith et al., 2022). Given our limited effects of HN001 on maternal care, the null results of Cowan et al. (2016) and Radford-Smith et al. (2022), and that early postpartum undisturbed maternal behaviors do not differ between germ-free mouse dams and controls with a normal microbiome (Sudo et al., 2004), we tentatively conclude that the gut microbiome has minor if any influences on postpartum caregiving activities in unstressed, female laboratory rodents.

Neurobiological mechanisms through which HN001 altered our postpartum anxiety-related and stress-coping behaviors are probably numerous. We focused on plasticity in three

monoamine systems (NE, DA, 5-HT) and found numerous probiotic effects in the PFC. HN001 alone was associated with lower PFC tissue levels of all three neurotransmitters and combined with chronic pregnancy stress associated with 5-HT and DA turnover. These results collectively suggest increased release and metabolism, rather than intracellular storage, of 5-HT and DA in response to treatment. These results should be considered in light of the methods used in our study, such that brains were collected within five minutes after the stressful FST. Early work found that DA and 5-HT levels or metabolism are not quickly affected in response to an acute stressor, although NE tissue content starts to fall within 3-15 minutes (Roth et al., 1982; Sudo et al., 1983; Paris et al., 1987; Suemaru et al., 1985), so NE levels even in our no-probiotic/no-stress controls may not have been as high as completely untested animals. Future work including untested animals would help confirm our monoamine results. Recent work by Huang et al. (2022) involving male mice found that whole-brain levels of NE, DA and 5-HT were reduced - and their metabolites increased - after chronic stress but that concurrent HN001 could prevent these effects. They had no unstressed, HN001-treated group to know if the probiotic alone would further increase these monoamines. A conceptually similar study in postpartum rats reported lower NE, DA and 5-HT the PFC in dams receiving chronic postpartum stress compared to unstressed controls, and that these reductions were also prevented by chronic *Lactobacillus casei* (Yang et al., 2022). In both studies, the probiotic treatments also prevented stress-related effects on affective behaviors. How 5-HT and DA release and receptor signaling in the PFC influence affective state is well studied and there is emerging evidence for the PFC's role in maternal caregiving (Lonstein et al., 2015). Other neurochemical systems worth future study include GABA, which is normatively upregulated postpartum and necessary for typical postpartum anxiety, depressive-like behaviors, and caregiving in unstressed rodents (Gilfarb and Leuner,

2022). Interestingly, male mice orally gavaged daily with *L. rhamnosus* (JB-1) for four weeks had altered expression of GABA receptor subunits in many brain sites, which was prevented by vagotomy (Bravo et al., 2011). Furthermore, adult male rats given both *L. rhamnosus* HN001 and a milk fat globule membrane-enriched product in their drinking water showed reduced expression of some GABA_A receptor subunit genes and the GABA transporter 3 in the amygdala (Dalziel et al., 2023).

A number of studies in adult humans and a few in other animals involving chronic administration of HN001, alone but more often combined with other probiotics, find changes in fecal microbial diversity and/or levels of particular microbes (e.g., Paturi et al., 2015; Vitellio et al., 2019; Huang et al., 2022; Lai et al., 2023; for exceptions see Kristensen et al., 2016; Lai et al., 2023; Young et al., 2022). Unlike some studies that do not appear to correct for multiple statistical comparisons, after we corrected for the many multiple comparisons made among our groups the only significant HN001 effects were to prevent the normative reduction in beta and alpha diversity occurring between pregnancy and postpartum in our control animals. This normative effect of pregnancy has been found before in rats (Ramsteijn et al., 2018), but not always (Pawluski et al., 2023), which is probably because of differences among studies in the particular time points sampled. When differences in diversity are found, reproductive state-specific hormone and metabolic profiles are probably responsible. Pregnancy stress also prevented the pregnancy-to-postpartum changes in diversity found in the controls, which is consistent with some work showing that pregnancy-stress increases or decreases fecal microbe alpha or beta diversity during particular periods of reproduction in rats (Jasaravec et al., 2017; Wang et al., 2023). The consequences of disrupting this normative change in microbial diversity could include driving the changes in brain and behavior we observed in our experimental groups.

In contrast to microbial diversity, our HN001 treatment did not significantly affect particular microbial phyla, families or genera. This is also surely because of the very high threshold for statistical significance required after correcting for multiple comparisons and also does not preclude the possibility that changes may have existed higher in the gastrointestinal tract (Young et al., 2022). Despite not reaching the threshold for statistical significance after correction, the most abundant microbes in our HN001-treated females were indeed members of the genus *Lactobacillus*, whereas in water-treated controls it was not, reassuring us that our treatment was reflected in the gut microbiome in an expected way.

In conclusion, chronic *L. rhamnosis* HN001 from early pregnancy through the postpartum period notably reduced postpartum anxiety behavior, which was associated with plasticity in prefrontal cortex monoamine levels. When combined with chronic variable stress in the last two weeks of pregnancy, HN001 also affected behavioral despair and increased DA and 5-HT turnovers in the prefrontal cortex. Lastly, probiotic and/or pregnancy stress prevented the normative postpartum reductions in fecal microbe diversity. These results indicate that chronic probiotic treatment can alter emotion-related behavior, brain neurochemistry, and the gut microbiome in reproducing females and provide intriguing implications for probiotic supplement use by pregnant and postpartum women. An important expansion upon these results in future studies would explore how HN001, with or without stress, affects maternal executive functioning (e.g., attention, impulsivity, memory) that critically modulates postpartum affective state and caregiving in both laboratory rodents and women (see Bridgett et al., 2017; Lonstein, Levy and Fleming, 2015; Gonzalez, 2015; Li, 2023).

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Figure Legends

Figure 1 – Experimental timeline. Preg = pregnancy, PP = postpartum.

Figure 2 – Measures of affective behaviors in laboratory rat dams chronically provided regular water or probiotic-treated water each night and/or given no stress or chronic variable pregnancy stress. A) Percentage of total entries (Mean \pm SEM) made into the open arms of an elevated plus maze, and B) Percentage of time spent (Mean \pm SEMs) spent in open arms, C) Percentage of total fluid intake (Mean \pm SEM) that was from the saccharin bottle during 1-hr preference test in, D) Latency (Mean \pm SEM) to float, and E) Percentage of time floating in a forced swim test.

*Significant main effect of stress, #Significant main effect of probiotic, $p < 0.05$, ^Significant stress by probiotic interaction, $p < 0.05$.

Figure 3 – Behaviors (Means \pm SEMs) displayed during undisturbed observations of maternal caregiving by postpartum rat dams chronically provided regular water or probiotic-treated water overnight and/or given no stress or chronic variable pregnancy stress. A) Behavioral fragmentation across test days (see text for statistical results), B) Largest behavioral fragmentation peak-minus-trough measurement, C) Frequency of being inactive away from pups, D) Frequency of self-grooming across test days (see text for statistical results). #Significant main effect of probiotic, ^Significant stress by probiotic interaction, $p < 0.05$

Figure 4 – Levels (Means \pm SEMs) ($\mu\text{g/g}$ tissue weight) of A) norepinephrine, B) dopamine, and C) serotonin in the prefrontal cortex of postpartum rats chronically provided regular water or probiotic-treated water overnight and/or given no stress or chronic variable pregnancy stress. D)

correlation between the percentage of entries mothers made into the open arms of the elevated plus maze and NA levels in their hypothalamus. Correlations between the percentage of time mothers spent in the open arms and prefrontal cortex levels of DA and 5-HT (E-F) and DA turnover (G). #Significant main effect of probiotic, ^Significant stress by probiotic interaction, $p < 0.05$.

Figure 5 - Fecal microbiome analysis. A) Beta diversity analysis using Principal Component Analysis (PCA) based on centered log-ratio-transformed RSV abundance and Aitchison distance matrix between 4 groups of samples during pregnancy (Preg) and postpartum (Postpart). Ellipses represent 80% confidence interval. The outer box plot shows projection of individual points onto PC1 and PC2 axis. Significance is marked with p-values on the outer box plot. Alpha diversity as measured by B) Shannon index and C) Observed species from fecal samples taken during late pregnancy and postpartum in female rats. Points in diamond shape represent mean abundance for that group of samples.

Figure 6 - A) Phylum, B) Family, and C) Genus stack bar charts for average percent abundance for topmost abundant taxa at the pregnancy and postpartum timepoints. D) Correlation between the percentage of time postpartum rats spent in the open arms of an elevated plus maze with their postpartum fecal levels of *Bacteroides dorei*.

Figure 1

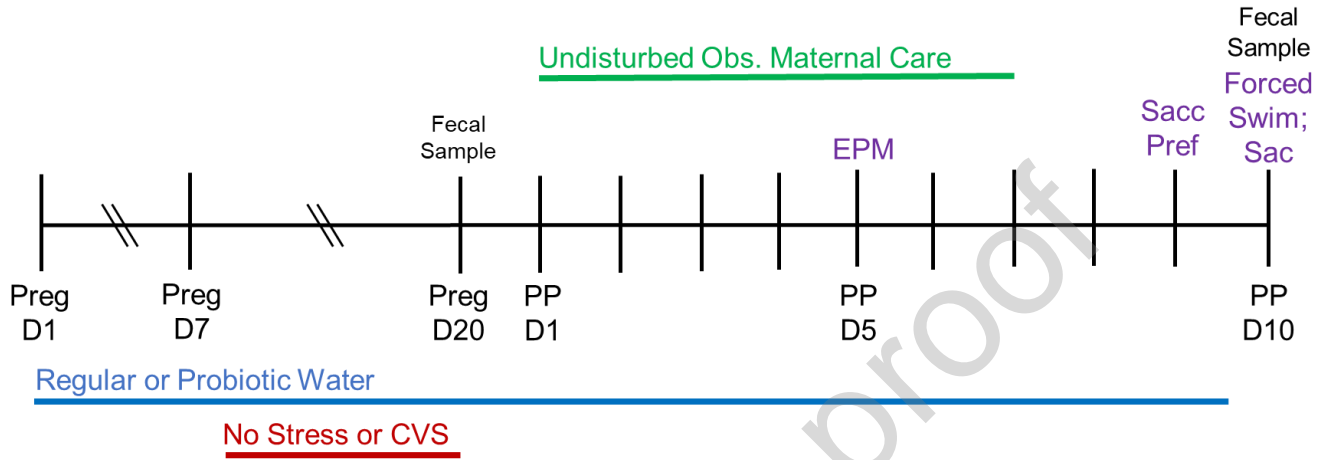


Figure 2

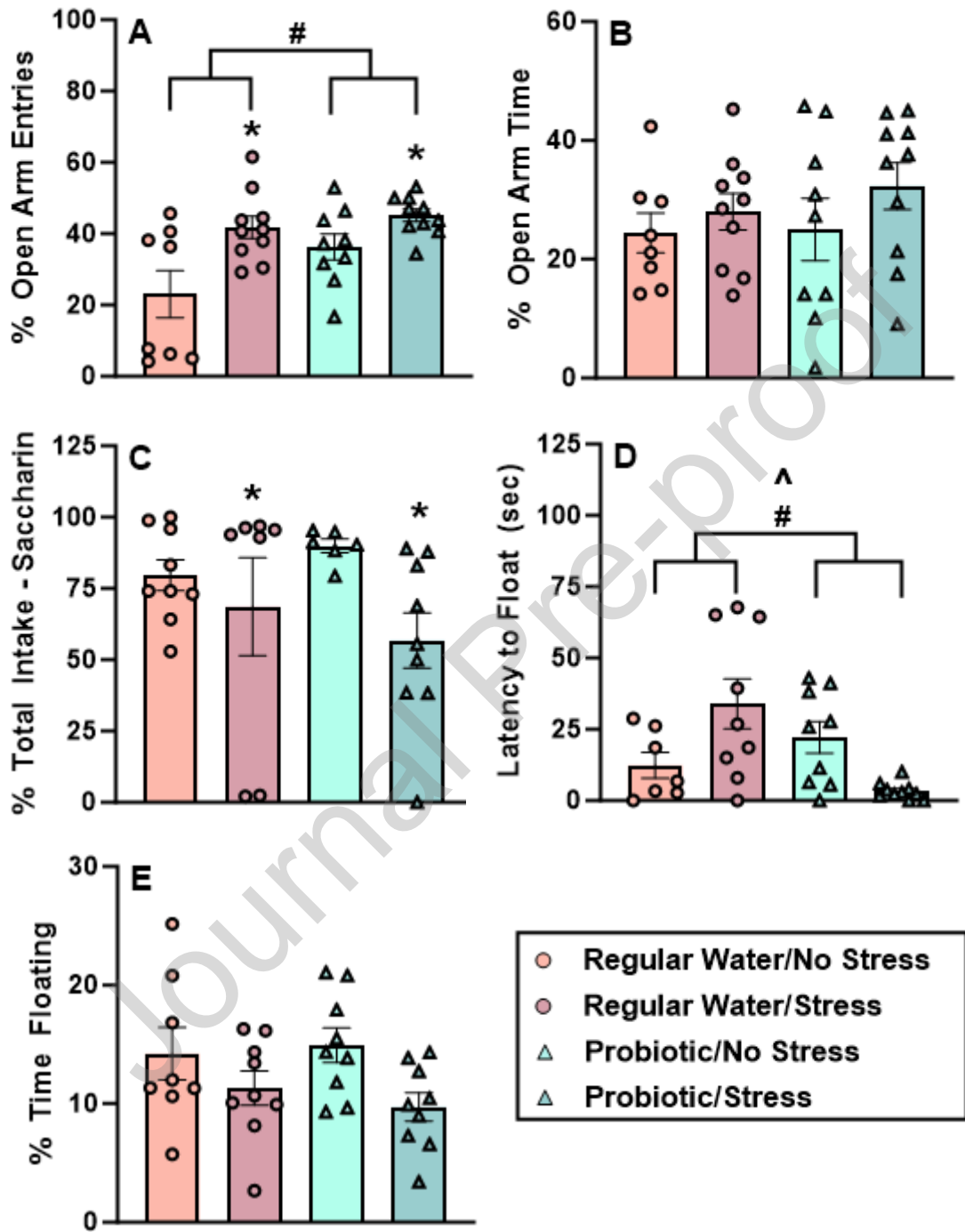


Figure 3

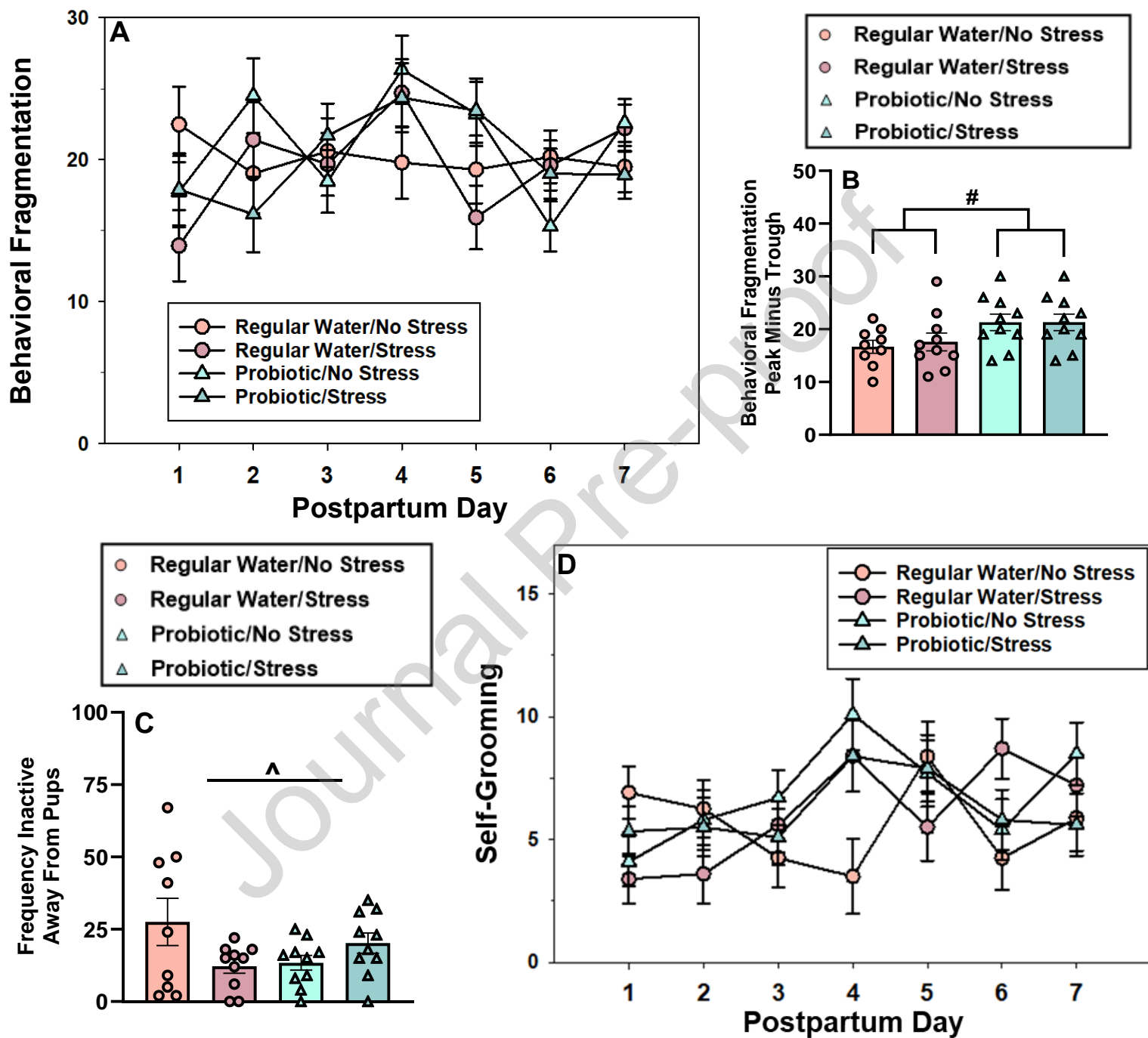


Figure 4

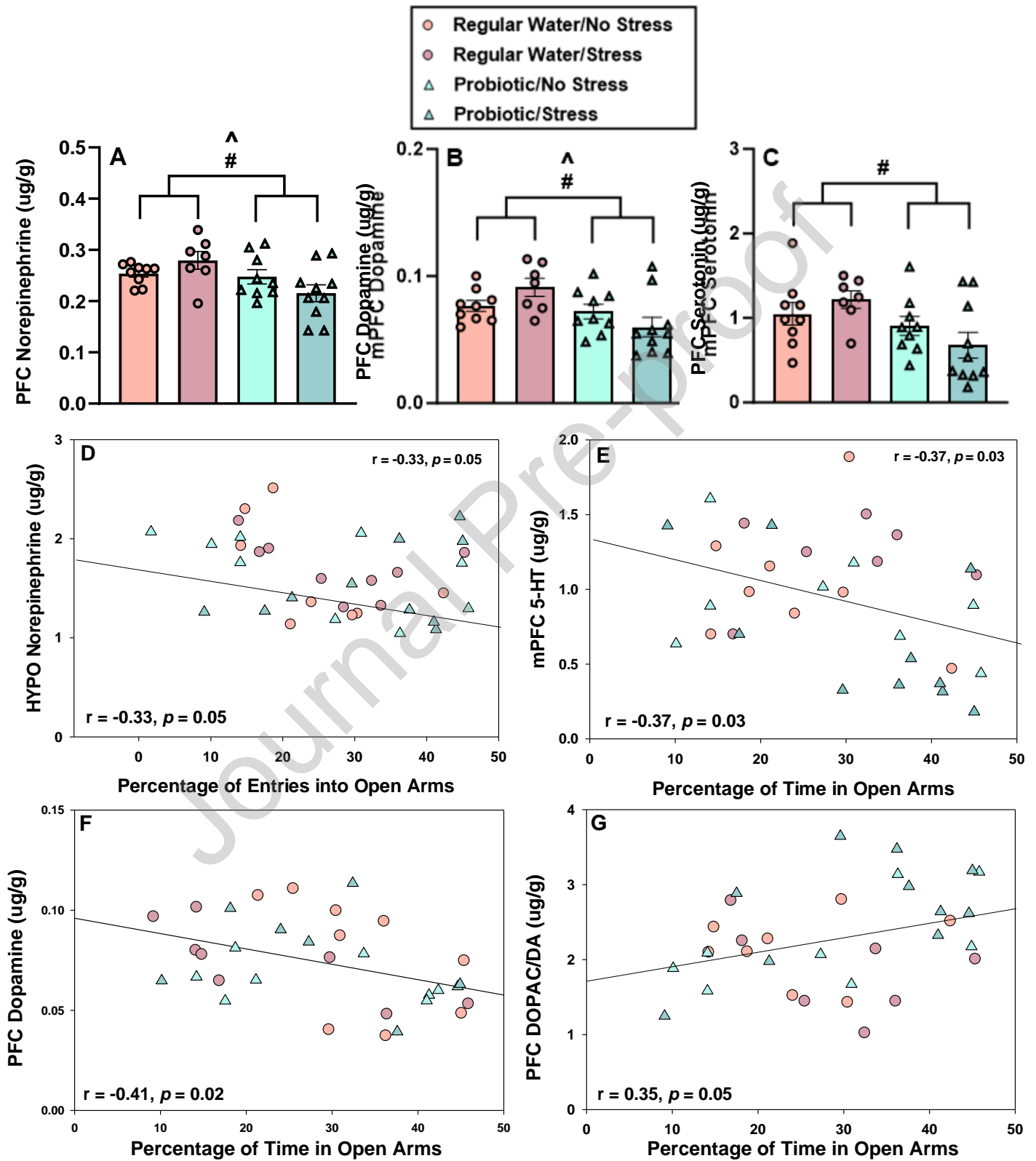


Figure 5

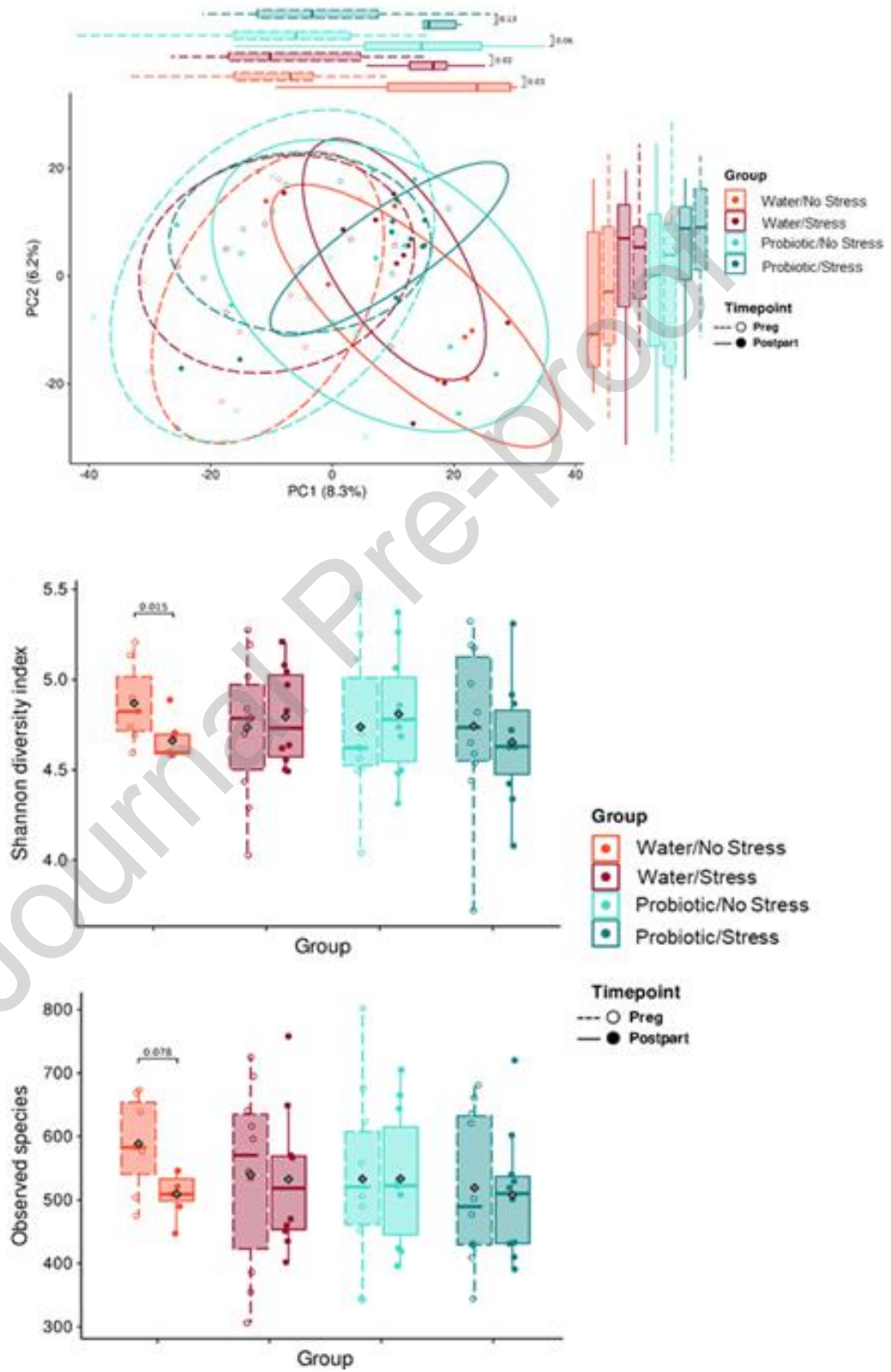
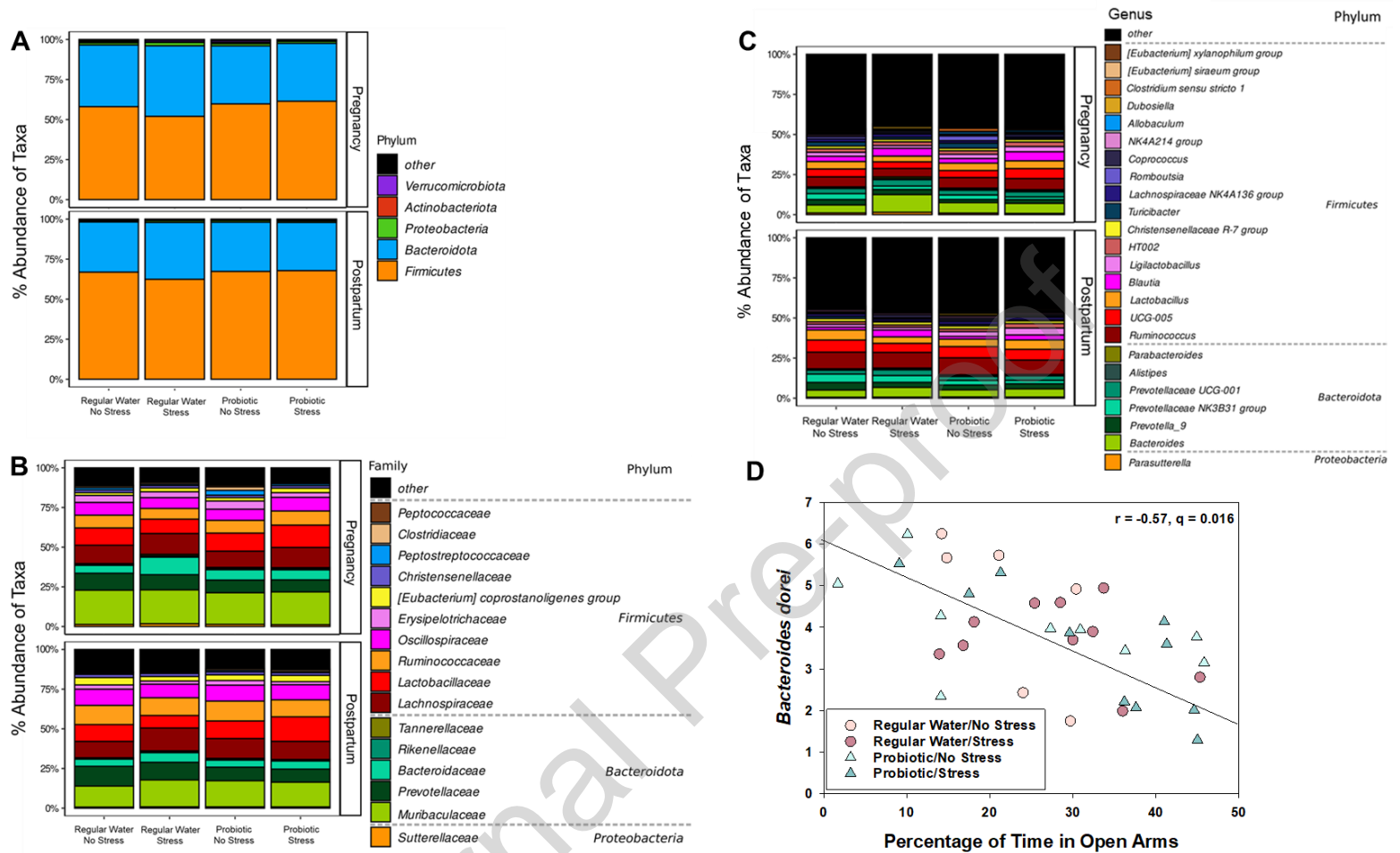


Figure 6



Declaration of interest statement: None.

Highlights:

- *Lactocaseibacillus rhamnosus* HN001 probiotic treatment reduced postpartum anxiety
- *L. rhamnosus* HN001 reduced prefrontal cortex norepinephrine, dopamine, & serotonin
- *L. rhamnosus* HN001 plus pregnancy stress reduced latency to float in forced swim
- *L. rhamnosus* HN001 or stress prevented postpartum reduction in microbial diversity
- Postpartum anxiety was negatively correlated with fecal *Bacteroides dorei*