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The social microbiome: *Eubacterium* links gut microbiota to prosocial behavior in stressed and naïve rats, a gut-brain axis study

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ABSTRACT

Background: Prosocial behavior is associated with positive health outcomes, but the underlying biological mechanisms remain unclear, especially regarding the role of the gut microbiome.
Results: We used the Helping Behavior Test to assess prosocial tendencies in rats and compared gut microbiome profiles between prosocial and non-social individuals across two experiments. In the first, we linked nucleus accumbens mRNA expression to microbiome composition in naïve rats. Prosocial behavior was associated with enriched *Eubacterium* species and genes tied to immune and neurotransmitter functions. An *in vitro* follow-up tested effects of additives on *Eubacterium ventriosum*. In the second experiment, we studied how early life stress (maternal separation) influenced prosocial behavior and the microbiome. Microbiome differences aligned with social behavior. Different stressors led to distinct microbiome profiles, especially among non-social rats, with variations in Bacillota and Bacteroidota abundance.
Conclusions: Overall, these detailed analyses provide insights into the behavioral, molecular, and microbial bases of prosocial behavior, highlighting the complex relationships between prosocial behavior, the gut-brain-microbiota axis, and early life experiences.

1. Introduction

Prosocial behavior, characterized as actions intended to benefit others, is fundamental to the functioning and well-being of individuals within communities (Penner et al., 2005). However, the manifestation of prosocial behavior is influenced by various internal and external factors, including stress. The relationship between stress and social behavior exhibits a complex pattern, displaying an inverted U-shaped relationship characterized by both negative and positive effects (Ben-Ami Bartal et al., 2016; Muroy et al., 2016). Particularly, arousal plays a vital role in empathic responses by facilitating the sharing and comprehension of the distress of others. Alongside environmental factors, certain biological factors, such as hormones (e.g., oxytocin and stress hormones) and specific brain regions, notably the nucleus accumbens (NAc), have been identified as pertinent to prosocial behavior (Hazani et al., 2025; Breton et al., 2022; Decety, 2011; Decety et al., 2016; Tankersley et al., 2007). Neural activity in the NAc was positively associated with social value orientation (Haruno et al., 2014), responds to prosocial calls in rats (Willuhn et al., 2014), and correlates with their ingroup helping behavior (Ben-Ami Bartal et al., 2021).

The microbiome has emerged as a critical modulator of host physiology and behavior, influencing immunity, metabolism, and neural development (Champagne-Jorgensen et al., 2020; Frankiensztajn et al., 2020; Glinert et al., 2022; Kayyal et al., 2020; Leclercq et al., 2017; Morton et al., 2023; Weiner et al., 2023). Preliminary studies reported associations between neural activity in the NAc and microbiome parameters (Dong et al., 2022; García-Cabrerizo et al., 2021). For example, NAc centrality levels in the neural network were associated with

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bacterial abundance in the context of obesity (Dong et al., 2022), substance use (Sens et al., 2023), and autism-like behavior in rats (Tevzadze et al., 2019).

The influence of the microbiome on social behavior is increasingly recognized (García-Cabrerizo and Cryan, 2024; Grinberg et al., 2022; Hayer et al., 2023; Sarkar et al., 2020; Uzan-Yulzari et al., 2024), with evidence that social relationships also shape microbial diversity (Baniel and Charpentier, 2024). Altered microbiota have been reported in individuals with social anxiety (Butler et al., 2023; Ritz et al., 2024), as well as in pigs and zebrafish exposed to social stressors (Nguyen et al., 2023; Scott et al., 2023). Studies using social defeat stress (SDS) have linked gut dysbiosis to social avoidance, with fecal microbiota transplantation or monocolonization reproducing social deficits (Agusti et al., 2024; Huang et al., 2025; Wang et al., 2024; Yadav et al., 2023). Moreover, microbiome depletion and dietary shifts impair social behavior in rodents (Reichelt et al., 2020; Sgro et al., 2024), and mother-calf separation in porpoises disrupts the microbiome and fecal metabolome (Shah et al., 2024).

The gut microbiome has bidirectional interactions with both stress response and social behavior, by affecting the development and function of the nervous system, as well as regulating immune responses (Bailey et al., 2011; Crumeyrolle-Arias et al., 2014; Foster et al., 2017; Kelly et al., 2016; Moeller et al., 2016; Morys et al., 2024; Osadchiy et al., 2019; Wu et al., 2021a). For example, diet- or antibiotic-induced microbiome alterations modulate oxytocin signaling and sociability (Buffington et al., 2016; Poutahidis et al., 2013; Tillisch et al., 2013; Uzan-Yulzari et al., 2024).

The microbiome also modulates responses to early life stress (ELS), influencing hormone release and gut-brain signaling (Ait-Belgnaoui et al., 2014; Donoso et al., 2020; Gareau et al., 2007). ELS models are accompanied by altered microbiota and social deficits that can be reversed with specific microbial manipulations (Kamimura et al., 2024; Mulder et al., 2024; Siddi et al., 2024; Tanabe et al., 2024; Wang et al., 2024).

In the present study, we investigated the complex interconnectedness of the gut-brain-microbiota axis, prosocial behavior, and stress. Based on emerging evidence suggesting possible associations between these factors, we hypothesized that variations in microbiome composition might account for some of the observed diversity in prosocial tendencies and could be simultaneously influenced by prosocial experiences. Moreover, we hypothesized that these microbial differences are linked to specific molecular changes, particularly within the NAc. Finally, we hypothesized that ELS would significantly affect prosocial behavior, microbiome composition, and the intricate relationship between the two.

To test these hypotheses, we used the "Helping Behavior Test" (HBT), a well-established animal model for exploring the underlying biological mechanisms of prosocial behavior (Kantor et al., 2025; Ben-Ami Bartal et al., 2021, 2011), where rats have the opportunity to release a trapped cagemate. Some rats reliably help ('openers'), while others do not ('non-openers'). The task itself elicits corticosterone responses, which may facilitate helping at an optimal level (Ben-Ami Bartal et al., 2016; Muroy et al., 2016). In Experiment 1, we compared microbiome composition and NAc transcriptomes between openers and non-openers and then used *in vitro* assays to test candidate modulators of the key microbial taxa. Additives were selected based on behavioral relevance (Gunaydin et al., 2014; Kiser et al., 2012; Liu and Wang, 2003; Moskowitz et al., 2001; Shahrokh et al., 2010), and their involvement in gut-brain interactions (Boyanova, 2017; Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Dave et al., 2016; Luczynski et al., 2016; Monstein et al., 2004; Ohlsson et al., 2006; Wang et al., 2021; Xie et al., 2022). In a second experiment, we investigated how ELS influences both microbiome composition and prosocial behavior (see Fig. 1 for the general timeline of the study).

Overall, this study presents a new perspective in the research of prosocial behavior, emphasizing its intricate connections with the gut-brain-microbiota axis and stress. The findings have the potential to enhance our understanding of the underlying mechanisms involved, providing valuable insights that may inform future research and therapeutic strategies for social disorders.

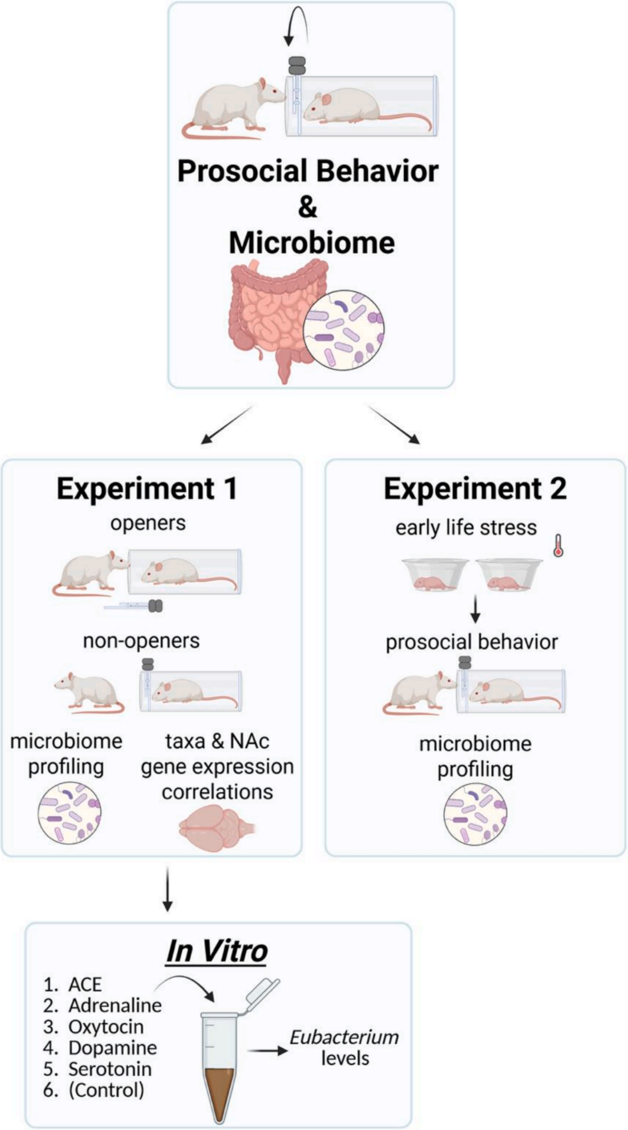


Fig. 1. General Timeline. Experiment 1 examined whether helping behavior status (opener or non-opener) was associated with the gut-brain-microbiome axis, using *in vivo* and *in vitro* set-ups and microbial and gene expression profiling. Experiment 2 further examined the effects of early life stress (maternal separation) on prosocial behavior and the microbiome. Abbreviations: ACE, Angiotensin Converting Enzyme; NAc, Nucleus Accumbens.

2. Materials and Methods

2.1. Animals

All studies included 10–11 week-old Wistar rats (Envigo RMS, Israel). In the first experiment, based on power analysis of the behavioral studies, 32 rats (16 males, 16 females) were purchased and housed in same-sex pairs under controlled experimental conditions. Within each pair, one rat was designated as the 'free' rat while other served as the 'trapped' rat in the HBT paradigm, as detailed below. Rats were provided unlimited food and water. The housing environment was maintained at a controlled temperature of 22 ± 2°C with a 12-hour light/dark cycle, with lights turning on at 0700. In the second experiment, for the ELS manipulation [Maternal Separation (MS); Huot et al., 2001; Lippmann et al., 2007], female rats were bred in our animal facility and housed individually during the last week of pregnancy. The stress experiment included: 16 control pairs, 16 Maternal Stress – Free (MS-F) pairs, 12 Maternal Stress – Trapped (MS-T) pairs, and 20 Vicarious Maternal Stress (VMS-F) pairs. The study protocol adhered to the guidelines set forth by the Society for Neuroscience and received approval from the Bar-Ilan University Institutional Animal Use and Care Committee (protocol no. 16–02-2018).

2.2. *Experiment 1 – In vivo phase*

2.2.1. *Behavioral test*

A week before the HBT (10–11 weeks of age), the rats were habituated to the experimental features and the experimenters for five days. This procedure aimed to minimize stress responses during the HBT. During this habituation phase, the rats were handled and placed in an empty testing arena (without a restrainer) for 30 min.

Helping Behavior Test. The HBT used the same protocol as previously published (Ben-Ami Bartal et al., 2011, 2021), and was conducted starting from age 11–12 weeks over 12 1-hour testing sessions on consecutive days except for Saturday. During each session, one rat (the trapped rat) was placed within a restrainer positioned at the center of a square (50X50 cm) testing arena. The restrainer had a custom-made door designed to be opened only from the outside. After placing the trapped rat in the restrainer, the other rat in each pair (the free rat) was introduced into the testing arena, and for 40 min it could potentially open the door and release the trapped rat. If the free rat did not open the door during the 40-minute period, the experimenter opened the door halfway, facilitating door opening by either the free or trapped rat. This procedure was implemented to prevent the development of learned helplessness and to ensure that the free rat learned that the door could be opened. On occasion, the trapped rat managed to open the door from the inside. In these cases, the rat was immediately placed back into the restrainer, and a plexiglass blocker was added to prevent it from reaching the door. At the end of the 12-day session, the rats were classified as either ‘openers’ or ‘non-openers’ based on their performances during the HBT. Rats that opened the door at least twice in the last three days of the testing period were classified as openers, while those that did not meet this criterion were categorized as non-openers, as previously described (Ben-Ami Bartal et al., 2011).

2.2.2. *Biological measures*

Microbiome. Feces were collected from the rats at two time points – three days before and two days after the HBT. By collecting feces on non-HBT experimental days, we avoided confounding the stress of feces collection with the HBT experiment. Feces were placed in empty tubes and frozen at −80℃ until analysis. Samples were processed to characterize the fecal microbiota with 16S rRNA gene sequencing as follows. We extracted DNA from all samples using the MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit, with bead plate (Thermo Fisher; Waltham, MA, USA) according to the manufacturer’s instructions and following a 2 min bead beating step. We then PCR-amplified the variable V4 region of the 16S rRNA gene using 515F-barcoded and 806R-non-barcoded primers (Caporaso et al., 2012). Each PCR reaction consisted of 25 μL PrimeSTAR Max PCR mix (Takara Kusatsu, Shiga, Japan), 2 μM of each primer, 17 μL of ultra-pure water, and 4 μL DNA template. Thermal cycler conditions were as follows: 35 cycles of denaturation at 98 °C for 10 sec, annealing at 55 °C for 5 sec, and extension at 72 °C for 20 sec, followed by a final elongation at 72 °C for 1 min. We purified amplicons using Kapa Pure magnetic beads (Roche; Basel, Switzerland) and quantified them using the Picogreen dsDNA quantitation kit (Invitrogen, Thermo Fisher; Waltham, MA, USA). We then pooled equimolar amounts of DNA from individual samples and sequenced the pool using the Illumina MiSeq platform at the Genomic Center at the Bar-Ilan University Azrieli Faculty of Medicine in Safed, Israel. Appropriate negative and positive controls were included. Data analysis is detailed below.

Brain Sectioning and RNA Extraction from NAc. Three days after the final HBT session, rats were euthanized by decapitation and brains were removed and frozen immediately on dry ice and then stored at −80℃ until sliced. Brain sections of the NAc from both hemispheres were located using a cryostat, according to the landmarks described in the ‘Rat Brain Atlas’ (Paxinos and Watson, 2006). Then, with a 1.5 mm Miltex biopsy punch plunger (Bar Naor, Israel), the NAc was extracted from 2.2 to 1.2 mm bregma. Tissue extractions were immediately frozen on dry ice. Total RNA was extracted from NAc brain punches using the QIAcube Connect (Qiagen; Hilden, Germany) with the RNeasy micro kit (cat no. 74004). For the RNA-seq analyses, only the right hemisphere punches were used. Samples were disrupted in 60ul RLT buffer (a lysis buffer) using plastic grinding rods. Then, 300ul more RLT buffer was added, and the samples were homogenized using a needle and syringe. The lysate was then loaded on the QIAcube Connect for automated extraction. The quality of the RNA was evaluated using a TapeStation 4200 (Agilent Tecnologies; Santa Clara, CA, USA) with the RNA kit (cat no. 5067–5576). The RIN values of all samples were in the range of 7.9–9.1, indicating high quality.

RNA-seq libraries were constructed simultaneously according to the manufacture’s protocol (NEBNext Ultra II Directional RNA Library Prep Kit for Illumina, cat no. E7760) using 250 ng total RNA as starting material. mRNA pull-down was performed using the Magnetic Isolation Module (NEB; Ipswich, MA, USA; cat no. E7490). After construction, the concentration of each library was measured using a Qubit (Invitrogen; Waltham, MA, USA) and the size was determined using a TapeStation 4200 with the High Sensitivity D1000 kit (cat no. 5067–5584). All libraries were mixed into a single tube with equal molarity. The RNA-seq data was generated on an Illumina NextSeq2000, using P2 100 cycles (Read1-100; Index1-8; Index2-8) (Illumina; San Diego, CA, USA; cat no. 20046811). Quality control was assessed for 100 bp reads using Fastqc (v0.11.5; Andrews, 2011); adapters and low-quality bases were trimmed using CUTADAPT (v1.12; Martin, 2011), and subsequent reads shorter than 20 base pairs were discarded.

2.3. *Experiment 1 – In vitro phase*

An *in vitro* experiment was conducted in which five additives [oxytocin, dopamine, adrenaline, serotonin, and angiotensin converting enzyme (ACE)] were chosen based on findings from our study (oxytocin, ACE, and adrenaline) or their already established relevance to social behavior (serotonin and dopamine; Gunaydin et al., 2014; Kiser et al., 2012; Y. Liu & Wang, 2003; Moskowitz et al., 2001; Shahrokh et al., 2010). The compounds were added separately to solutions of individual fecal samples (resuspended in PBS) collected from 6 male and 6 female naïve rats, respectively. One mg of dopamine, adrenaline, or serotonin was dissolved separately in 1.5 ml of PBS. 0.2 mg of oxytocin was dissolved in 1 ml of PBS, and 0.5 units of ACE were diluted in 50 μl PBS. One hundred μl of each solution (oxytocin, dopamine, adrenaline, serotonin) was added, respectively, to tubes containing a 1 ml PBS-fecal slurry suspension. In the case of ACE, 10 μl was added. All concentrations were selected based on their average reported levels in plasma (Al-Qattan et al., 2016; Ben-Jonathan et al., 1977; Elabd et al., 2008; Kutlu et al., 2010; Liu et al., 2015; Lv et al., 2020; Pertsch et al., 1993; Thomas et al., 1989; Tokui et al., 2021). Control samples consisted of the fecal slurry with an addition of 1 ml of PBS. In total, the five substances and a control were each added, separately, to 6 male and 6 female fecal sample slurries. The samples were incubated under anaerobic conditions, at 37 °C with constant shaking for 24 h. Then DNA was immediately extracted using a MagMAX Ultra Nucleic Acid Isolation Kit (Thermo Fisher, Waltham, MA) like above.

Quantitative real-time PCR (qRT-PCR). Abundance of *Eubacterium ventriosum* (taxid: 39496; Schoch et al., 2020), chosen retrospectively, in total stool samples was determined by qRT-PCR analysis. qRT-PCR with appropriate primers (Table 1) was performed with FAST SYBR Green master mix (Applied Biosystems; Waltham, MA, USA; cat no. B-4385612) in triplicates using the ViiA7 real-time PCR system (Applied Biosystems). In order to quantify total bacterial load, qRT-PCR of the 16S rRNA gene was performed (Table 1); the PCR protocol was 95 °C for 3 min, followed by forty cycles of denaturation at 95 °C for 3 sec, and annealing/elongation at 64 °C for 30 sec. For *E. ventriosum*, a different program was used: 95 °C for 10 min, with forty cycles of denaturation at 95 °C for 10 sec, and annealing/elongation at 60 °C for 30 sec. Primer specificity was verified by running qRT-PCR products in 3 % agarose gel and visualizing appropriate bands. Results were quantified as ΔΔ Ct with the 16S rRNA gene as an endogenous control.

2.4. Experiment 2

2.4.1. ELS manipulation

Maternal Separation. The MS procedure was conducted from postnatal day 1 to 14, excluding Saturdays, in accordance with established protocols (Huot et al., 2001; Lippmann et al., 2007). The MS procedure was performed in three batches, comprising a total of twenty litters. Within each batch, litters were randomly classified to stress (MS) or ‘vicarious stress’ (VMS; bystander) litters. Overall, 10 litters were utilized for the MS condition and 10 for the VMS condition. From these litters, 16 female pups were assigned to the MS-F group, 12 female pups to the MS-T group, and 20 female pups to the VMS-F group (approximately 2–3 pups per litter). Before taking the pups for the MS procedure, the dams were removed from home cages. Litters were then moved to another room, and each pup was placed individually in a small box with holes, within a 33 °C incubator, for 3 h. The ‘vicarious stress’ litters remained in their home cage, with their dam, directly adjacent to the MS cages. This arrangement allowed for exposure to the stressful conditions experienced by the stressed litters without direct separation from their dam. Female offspring were subsequently weaned on postnatal day (PND) 21 and raised in pairs of the same experimental group until the HBT procedure. All efforts were made to ensure animal welfare during the MS procedure. The control rats ($n = 16$) were in separate rooms and underwent the HBT at different times to prevent the influence of odor marks in the behavioral experimental room.

2.4.2. Behavioral test

Helping Behavior Test. The HBT protocol was the same as described in Experiment 1. The current experiment encompassed four conditions: (1) Control group: Neither the free rats nor the trapped rats underwent the MS procedure, nor were they exposed to the stressed rats; (2) MS-F: The free rats were stressed as infants, while the trapped rats remained naïve to this stress manipulation; (3) MS-T: The trapped rats were stressed as infants, while the free rats remained naïve to this stress manipulation; and (4) VMS-F: The free rats were exposed to the stressed rats in adjacent cages from the day they were born, serving as bystanders to the maternal stress procedure, while the trapped rats remained naïve to this stress manipulation (Fig. 5**A**).

Table 1
Primers used for qRT-PCR

Gene	Forward primer	Reverse primer
16S (Huber et al., 2007)	111-967F-PP: CNACGCGAAGAACCTTANC 112-967F-UC3: ATACGCGARGAACCTTACC 113-967F-AQ: CTAACCGANGAACCTYACC 114-967F-S: CAACGCGMARAACCTTACC	115-1046R-S: CGACRRCCATGCANCACCT
Eubacterium ventriosum (NCBI Nucleotide Database, 2019)	ACATTGGGACTGAGACACGG	CGTATTTAGCCGGGGCTTCT

2.4.3. Biological measure

Microbiome. Stool collection and 16S rRNA gene sequence profiling were carried out as described in Experiment 1.

2.5. Statistical analysis

Behavioral Measures. Results are displayed as means ± standard error of the mean (SEM). Data were analyzed with SPSS (IBM, version 28.0). The door opening measure was analyzed with univariate analysis of variance (ANOVA) followed by Bonferroni pairwise post-hoc tests. Chi-square tests of independence were used when comparing groups for proportion of openers. All tests were two-tailed with a significance level set at $p < 0.05$.

Microbiome Analysis. For uniformity, all analyses were conducted only on the paired samples (animals for which both the ‘before’ and ‘after’ microbiome samples were collected and passed quality control). For the first experiment (openers vs. non-openers) we analyzed the microbiota from 16 animals with paired pre- and post-HBT samples (32 samples total): 8 openers (2 females, 6 males) and 8 non-openers (4 females, 4 males). The stress experiment included microbiota analysis from 48 animals (96 samples total): 5 control, 14 MS-F, 9 MS-T, and 20 VMS-F rats. The 16S rRNA gene sequence data were initially processed with QIIME2 version 2020.8 (Bolyen et al., 2019) using default parameters. We used DADA2 (Callahan et al., 2016) to filter noisy sequences, correct sequencing errors, remove chimeric sequences, remove singletons, and dereplicate sequences into amplicon sequence variants (ASVs). Taxonomy was assigned using classify-sklearn naïve bayes classifier against GreenGenes (DeSantis et al., 2006) and Silva138 (Quast et al., 2013) databases. After the QIIME2 pipeline, downstream analysis was performed using the phyloseq (version 1.34.0) R/bioconductor package for handling and analysis of high-throughput phylogenetic sequence data (McMurdie and Holmes, 2013). The taxonomy was first cleaned, and empty taxa were filtered out, using only those which appeared in at least three samples. The samples were then rarefied (using rarefy_even_depth function) to a minimum sequence depth of 9,500 and scaled by relative abundance.

We examined patterns of alpha and beta diversity for different groups of samples. Alpha diversity was calculated using Faith’s PD (Faith, 1992), and beta diversity was calculated using the weighted UniFrac measure (Lozupone and Knight, 2005). To assess gut microbiome differential abundance, DESeq2 was used (version 1.36.0; Love et al., 2014), and significant taxa were identified (threshold of adjusted p values < 0.05 and |log2foldchange|>=0.58). In experiment 1, sex was used as a blocking factor in the openers analysis. Heatmaps were generated using pheatmap (Kolde, 2019), and bar plots were generated in R using ggplot2 (Hadley, 2016). The taxa with differential abundances, as identified by DESeq2 were further compared across all experimental groups by analysis of variance with Tukey’s post-hoc tests.

RNA Sequencing Data Analysis. For RNA sequencing data analysis, we used 8 NAc samples: 5 openers (2 females, 3 males) and 3 non-openers (1 females, 2 males). Reads were aligned to the *Mus musculus* reference genome GRCm38 using STAR (version 020201; Dobin et al., 2013), and quantification of reads was performed using htseq-count (version 0.12.4; Anders et al., 2015) on a list of genes (Ensembl gtf file; Howe et al., 2021). Differential gene expression analysis was then performed using the DESeq2 (version 1.30.1) R/bioconductor package (Love et al., 2014). Significant differentially expressed genes were selected using threshold values of adjusted p-value smaller than 0.05 and |log$_2$fold change| greater or equal to 0.58. The volcano plot was rendered using ggplot2 (version 3.4.2; Hadley, 2016). The heatmap was rendered using pheatmap (version 1.0.12). For pathway enrichment analysis, Metascape (metascape.org; Zhou et al., 2019) was used to analyze differentially expressed genes. Additionally, Gene Set Enrichment Analysis (GSEA version 4.3.2; Subramanian et al., 2005) was used for all the genes ranked ($-\log_{10}(\text{pvalue})/\text{sign}(\log_2\text{FoldChange})$) using three datasets: hallmark, Curated Canonical Pathways, and GO gene sets (Mootha et al., 2003; Subramanian et al., 2005). GSEA results (q-value<=0.25 as per suggested program defaults for this exploratory analysis) were categorized and plotted (using ggplot2 version 3.4.2). Next, gut microbial genera were correlated with NAc significant differentially expressed genes from their respective overlapping samples, using Spearman correlations on significant gene expression normalized counts and microbial taxa normalized abundance with the corr.test function from the psych R package (version 2.2.9; Revelle, 2017) for each gene-taxon pair. Significant gene-taxa correlations (Spearman's correlation adjusted p-value $\leq$ 0.05) were visualized using corrplots (version 0.92; Wei & Simko, 2021). A scatter plot was also rendered for each gene-taxon pair using ggscatter from the ggpubr R package (version 0.5.0; Kassambara, 2018). Given the current study's aim to investigate the gut-brain axis in the context of prosocial behavior, in the results section we focused on those genes that correlated with the microbiome data.

***In Vitro* Phase Analysis.** Data were visualized and statistical significance calculated with GraphPad Prism (Windows, version 9.5); presented as mean ± SEM. One-way ANOVA followed by Tukey-Kramer's post-hoc test was used to compare qRT- PCR results.

3. Results

3.1. *Experiment 1: In Vivo Phase. Differences between Openers and Non-Openers*

3.1.1. *Openers expressed a stable social behavior pattern*

Following the HBT, rats were classified as either 'openers' or 'non-openers' based on the performance of the free rats in the HBT (Fig. 2**A**). 43.75 % (14/32) of the free rats consistently opened the door and were classified as openers, while the remaining 56.25 % (18/32) were classified as non-openers. There was no significant difference between females and males in the number of rats classified as 'openers' [female openers: 6/16, male openers: 8/16; $\chi^2(1) = 0.5079$, $p = 0.4760$]. Overall, a significant difference was observed between openers and the non-openers in the latencies to door opening across days [$F(1,30) = 663.3$, $p < 0.0001$; Fig. 2**B,C**]. Statistical analysis did not reveal significant differences between experimental batches in either the number of doors opened by the rats [$F(2,29) = 0.003$, $p = 0.9975$], or the proportion of rats classified as openers (Batch 1: 6/12 openers; Batch 2: 5/12 openers; Batch 3: 3/8 openers; $\chi^2(2) = 0.3386$, $p = 0.8442$). Of these subjects, we characterized the microbiota of 16 animals for whom both before and after HBT feces samples were available (32 samples total). There were no behavioral differences between the rats included in the microbiome analyses and those who were not [latencies to door opening; openers: $F(1,12) = 1.874$, $p = 0.196$, non-openers: $F(1,16) = 0.3832$, $p = 0.545$; **Fig. S1**].

3.1.2. *Prosocial rats exhibit enriched Eubacterium in their gut microbiota*

To explore links between the gut microbiome and prosocial behavior, we conducted 16S rRNA gene sequencing. No significant differences in microbiome composition were found between sexes, nor was there an interaction between sex and opening status. Alpha and beta diversity did not differ between groups or time points. DESeq2 analysis was conducted separately for 'before' and 'after' samples. Before the test, there were no significant differences between the taxa of the two groups. After testing, however, three taxa significantly differed between openers and non-openers: *Eubacterium ventriosum, Eubacterium CAG-180,* and unclassified *COE1* (Fig. 2**D**). *E. ventriosum* and *E. CAG-180*, both members of the *Eubacterium* genus, were found to be over-represented in the openers, while *COE1* was under-represented.

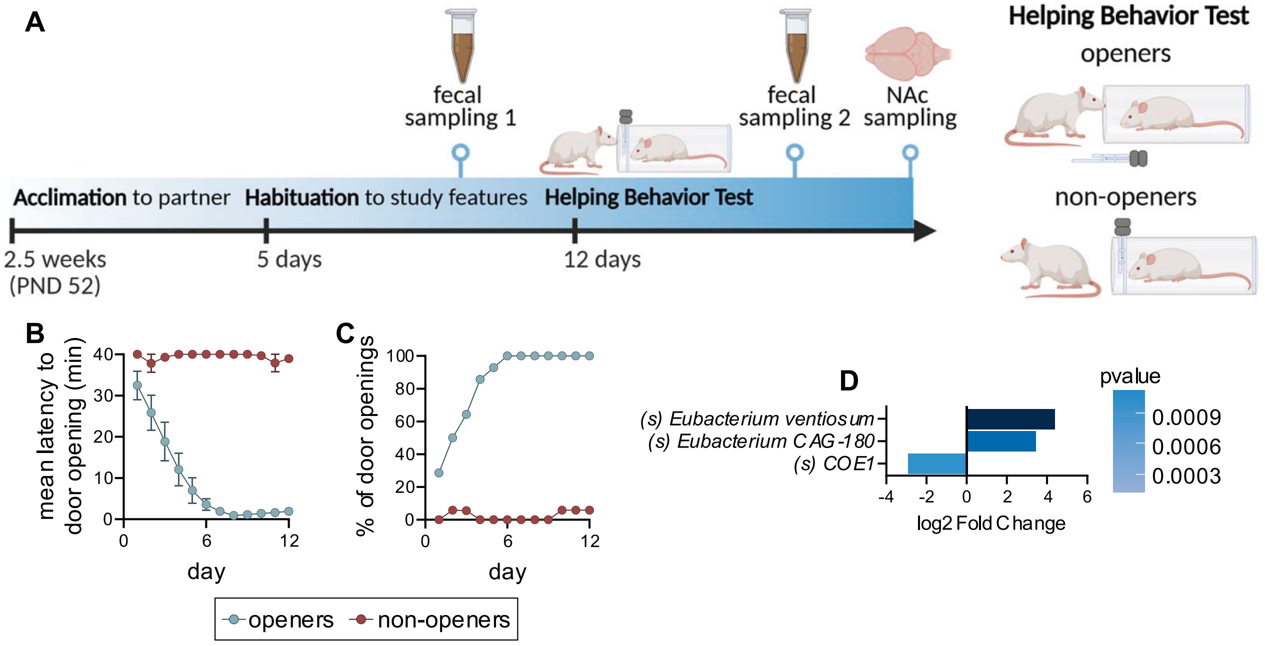


Fig. 2. Different Prosocial Patterns in Naïve Rats. (A) Experimental timeline for experiment 1. (B) During the helping behavior test (HBT), latencies to door opening decreased over time only in the opener group (two-way mixed ANOVA followed by Bonferroni's post-hoc test; n = 16 per group; data are presented as means ± SEM). (C) Percent of door openings over the 12 days of the HBT. Rats show consistent behavioral patterns for door opening as openers and non-openers, respectively (n = 16 per group). (D) Bar plot depicting microbes relatively enriched or decreased in the naïve openers compared to non-openers (DESeq2, n = 8 per group). Left bars represent enrichment in the openers group. Bar color (darkness) represents q-value.

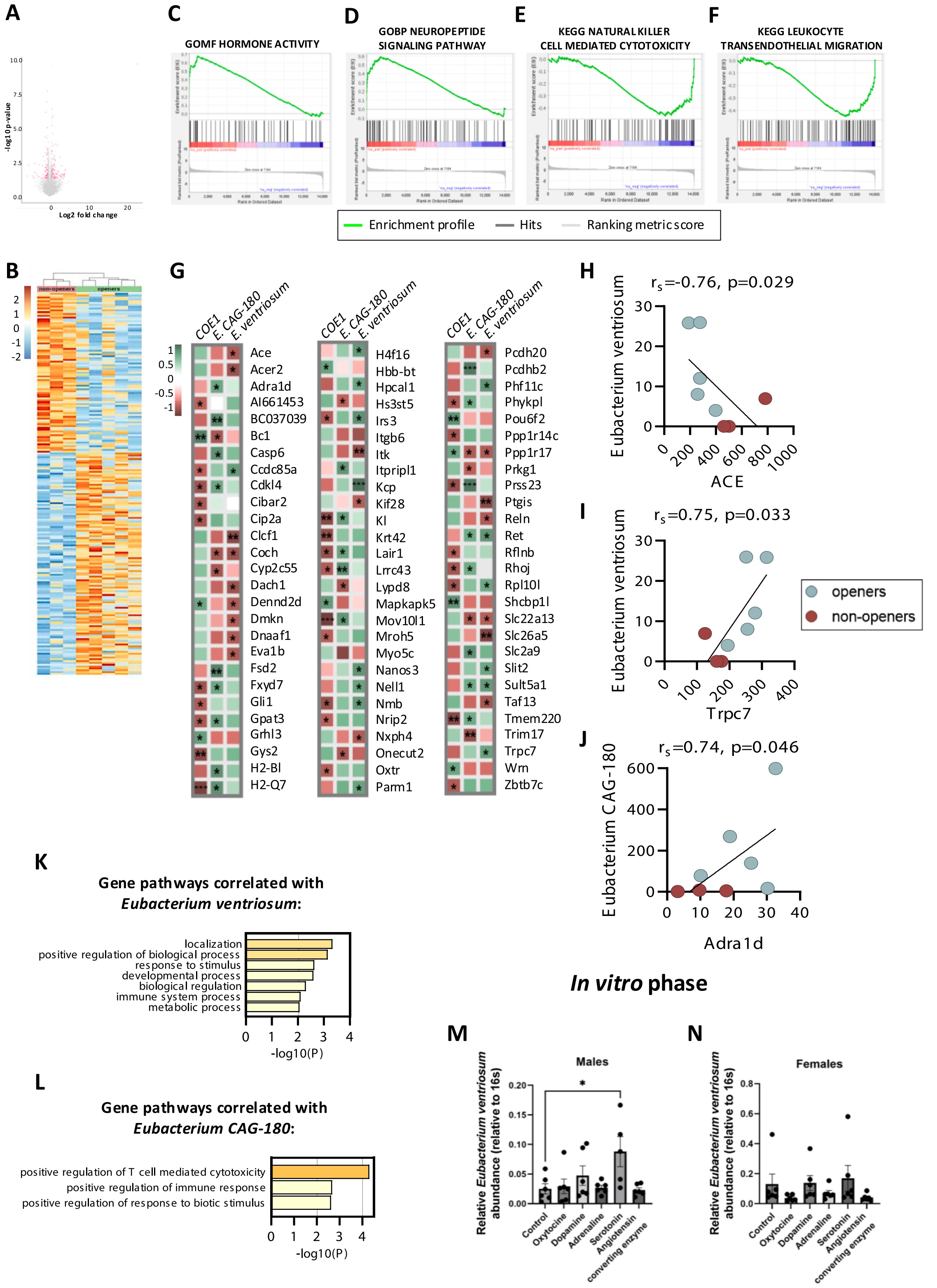


Fig. 3. Genomic Differences in the NAc between Openers and Non-Openers. (A,B) Volcano plot and heatmap for normalized gene counts that differ significantly between openers and non-openers (RNA-seq; openers: n = 5, non-openers: n = 3). (B) Z scores are represented by the color gradient. (C,D) Gene set enrichment analysis (GSEA) of pathways enriched in the openers. (E,F) GSEA of pathways decreased in the openers group. (G) Heatmaps of genes significantly correlated with one or more of the taxa *COE1*, *Eubacterium CAG-180*, and *Eubacterium ventriosum*. The direction and strength of the correlation is indicated by the color gradient, and the significance of the correlation is indicated with an asterisk(s) (Spearman's correlation; n = 8). (H,I) Correlations between relative abundance of *ACE* (H) and *Trpc7* (I) with *Eubacterium ventriosum*. (J) Correlation between *Adra1d* and *Eubacterium CAG-180*. (K,L) Metascape analysis demonsrates pathways of genes correlated with *Eubacterium ventriosum* (K) and *Eubacterium CAG-180* (L). (M,N) Abundance of *Eubacterium ventriosum* following the addition of oxytocin, dopamine, adrenaline, serotonin, and angiotensin converting enzyme to fecal samples from naïve male (M) or female (N) rats (one-way ANOVA followed by Tukey-Kramer's post-hoc test; n = 5–6; *$p < 0.05$).

3.1.3. *Eubacterium* is associated with NAc – gut-brain-microbiota axis

RNA-seq analysis revealed transcriptional differences between openers and non-openers in NAc (Fig. 3**A**). 208 genes were significantly differently expressed in the two groups, including *Oxtr*, *Adra2a*, *Adra1d*, and *Trpc7*, which were found to be upregulated in openers, and *ACE*, which was found to be downregulated (Fig. 3**B**). Several neural pathways differentiated between openers and non-openers, including calcium, glutamatergic, and locomotion pathways, which were upregulated in the openers group (**Fig. S2**). GSEA further showed that several pathways were associated with helping behavior, including upregulation of 'hormone activity' and 'neuropeptide signaling' pathways (Fig. 3**C,D**) and downregulation of 'natural killer cell mediated cytotoxicity' and 'leukocyte transendothelial migration' pathways (Fig. 3**E,F**).

From the genes that distinguished openers and non-openers, 35 genes significantly correlated with *E. ventriosum* abundance, and 34 genes significantly correlated with *E. CAG-180* (Fig. 3**G;** for statistics see **Table S1**). For example, *ACE* and *Trpc7* were significantly correlated with *E. ventriosum* (r_s = -0.76, p = 0.029; r_s = 0.75, p = 0.033 respectively; Fig. 3**H,I**). *Adra1d* was positively correlated with *E. CAG-180* (r_s = 0.74, p = 0.046; Fig. 3**J**). Moreover, genes correlated with *Eubacterium* taxa converged into several pathways, including localization, response to stimulus, and immune system processes (Fig. 3**K,L**).

Several immune-related genes were differentially expressed between openers and non-openers, including *Itk*, *Clcf1*, *Itgb6*, *Ptgis*, *Evan1b*, and *Nell1*, all of which were significantly correlated with *E. ventriosum*. Additionally, *H2-Bl* and *H2-Q7* differed between groups and were positively correlated with *E. CAG-180* (**Table S1**). At the pathway level, we found that the 'acute inflammatory response' pathway was upregulated in openers, while the 'acute-phase response' and 'cytokine production' pathways were downregulated. GSEA further showed that immune-relevant pathways were downregulated in openers (Fig. 3**E, F**). Additionally, the taxon *E. ventriosum* was associated with the gene pathway 'immune system process', and *E. CAG-180* was associated with 'positive regulation of T cell mediated cytotoxicity' pathway (Fig. 3**L**).

3.1.4. Effects of in vitro serotonin enrichment on fecal microbiota *E. ventriosum* levels in male rats

Based on *in vivo* findings, we conducted an *in vitro* assay testing five additives—oxytocin, serotonin, dopamine, adrenaline, and ACE—on *E. ventriosum* levels in cultured gut microbiota. Compared to PBS controls, serotonin significantly increased *E. ventriosum* abundance in samples from male rats [F(3,24) = 3.371, p < 0.05; post-hoc for serotonin: p < 0.05; Fig. 3**M**], but not in those from females (Fig. 3**N**). The remaining additives did not have a significant influence on the levels of *E. ventriosum in vitro*.

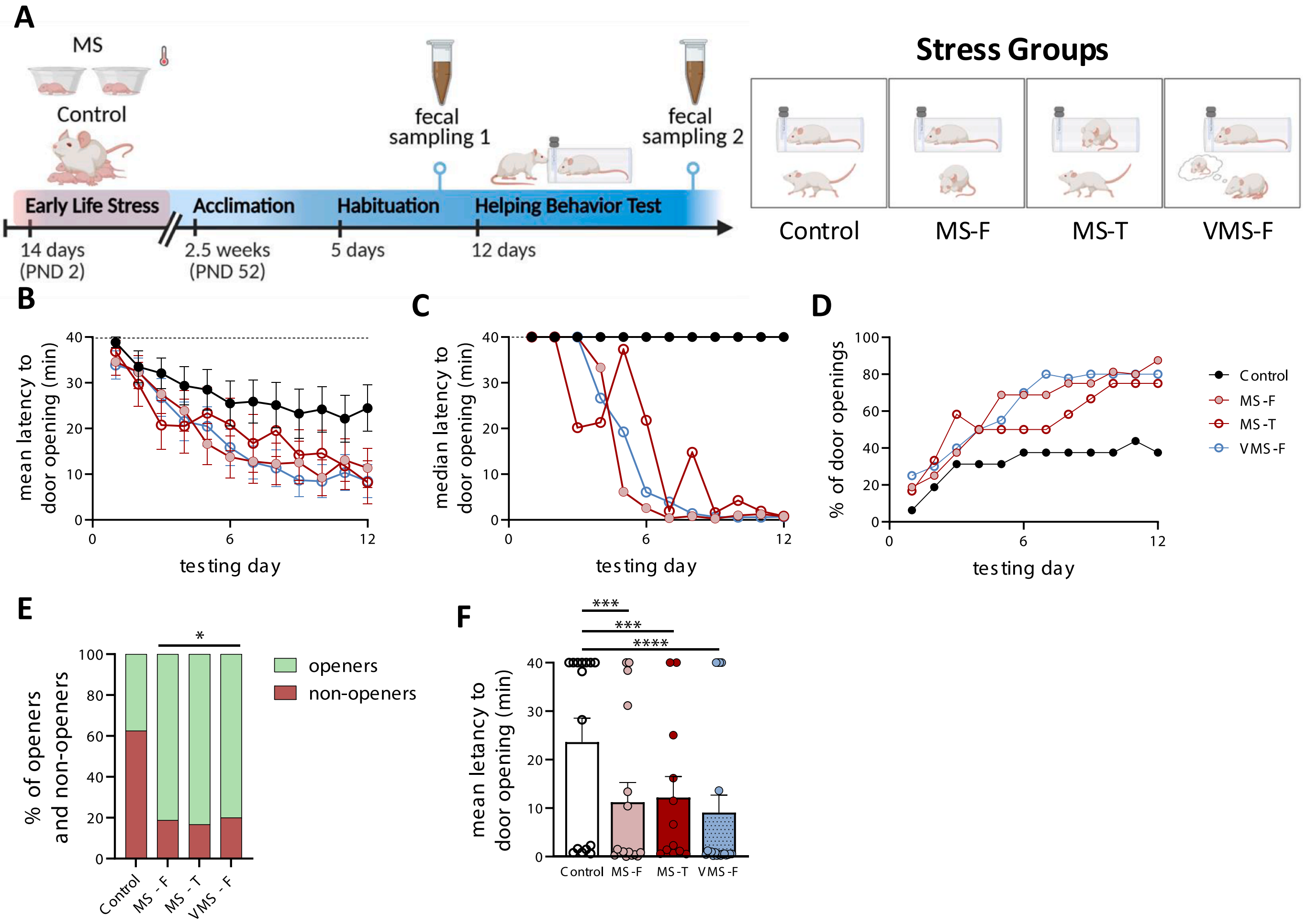
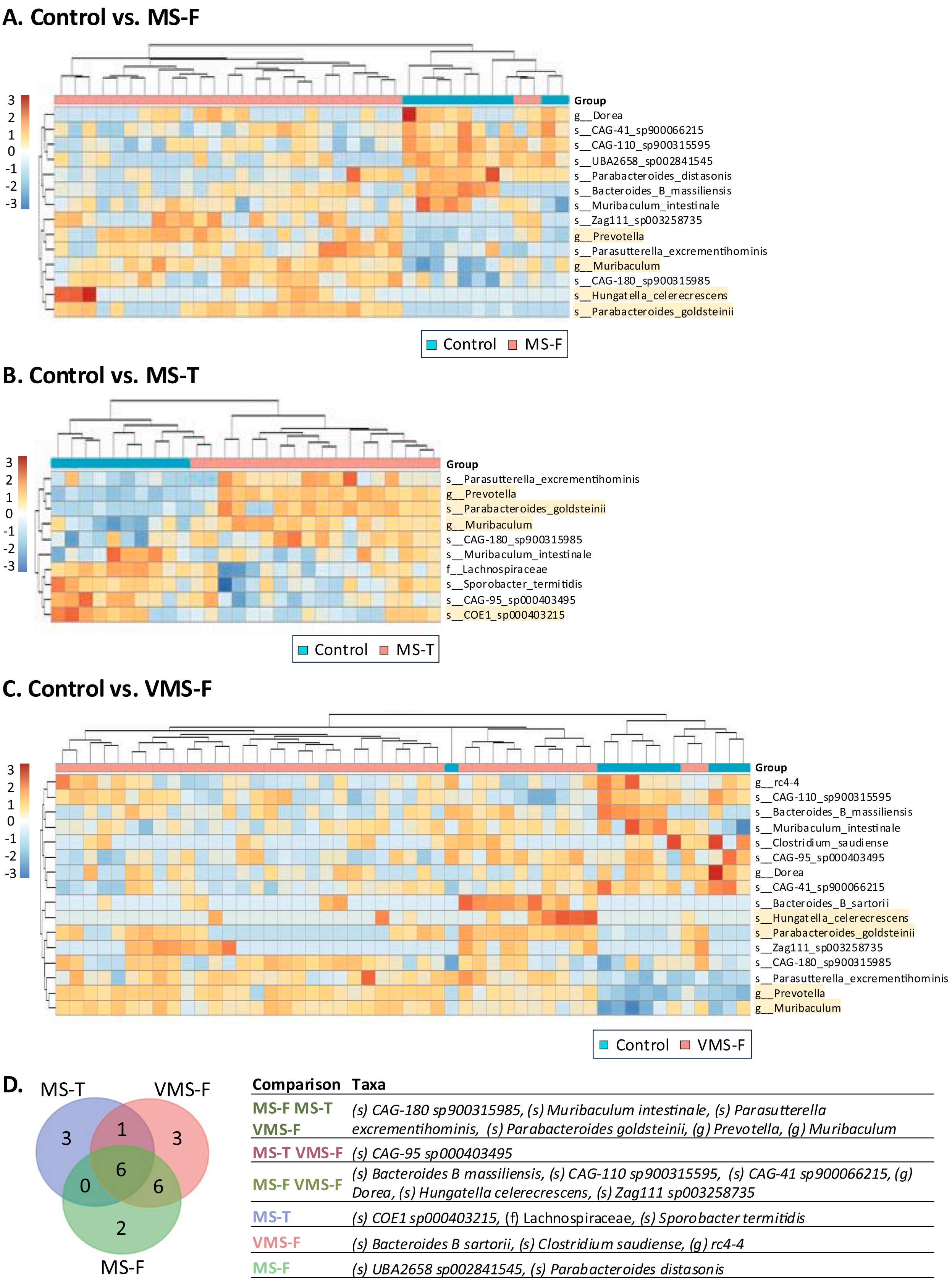


Fig. 4. Stress Induced Differences in Prosocial Behavior. (A) Experiment 2 timeline and group descriptions. (B) During the helping behavior test (HBT), mean latencies to door opening decreased in all the groups (two-way mixed ANOVA followed by Bonferroni's post-hoc test). (C) Median latencies to door opening over the HBT days show noticeable decreases in the stress groups. (D) The percent of door opening increased over the HBT days. (E) The percent of openers was significantly higher in all stress groups (merged for this analysis) compared to the control group (Chi-squared test). (F) On the last three days of the HBT, latencies to door opening were significantly lower in the stress groups compared to control (one-way ANOVA followed by Bonferroni's post-hoc test). Data are presented as means ± SEM (n = 16 in control, n = 16 in MS-F, n = 12 in MS-T, and n = 20 in VMS-F). *p < 0.05, ***p < 0.001, ****p < 0.0001. Abbreviations: PND, post-natal day; MS, Maternal Separation; MS-F, Maternal Separation Free; MS-T, Maternal Separation Trapped; VMS-F, Vicarious Maternal Separation Free.



Comparison	Taxa
MS-F MS-T VMS-F	*(s) CAG-180 sp900315985, (s) Muribaculum intestinale, (s) Parasutterella excrementihominis, (s) Parabacteroides goldsteinii, (g) Prevotella, (g) Muribaculum*
MS-T VMS-F	*(s) CAG-95 sp000403495*
MS-F VMS-F	*(s) Bacteroides B massiliensis, (s) CAG-110 sp900315595, (s) CAG-41 sp900066215, (g) Dorea, (s) Hungatella celerecrescens, (s) Zag111 sp003258735*
MS-T	*(s) COE1 sp000403215,* (f) Lachnospiraceae, *(s) Sporobacter termitidis*
VMS-F	*(s) Bacteroides B sartorii, (s) Clostridium saudiense, (g) rc4-4*
MS-F	*(s) UBA2658 sp002841545, (s) Parabacteroides distasonis*

Fig. 5. Stress Induced Changes in Microbiome Profiles. (A-C) Heatmaps of the normalized taxa abundances that differ significantly between control and MS-F (A), MS-T (B), and VMS-F (C) rats, respectively. Z scores are represented by the color gradient. The four most significant taxa are marked in yellow. (D) A Venn diagram demonstrates the overlapping significantly differentially abundant taxa in the three comparisons between the stress groups to control (n = 10 in control, n = 28 in MS-F, n = 18 in MS-T, and n = 40 in VMS-F). Abbreviations: Maternal Separation Free; MS-T, Maternal Separation Trapped; VMS-F, Vicarious Maternal Separation Free. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Experiment 2: The Effects of Stress on Prosocial Behavior and the Microbiome

3.2.1. Direct and indirect stress increases prosocial behavior

To examine the effects of MS on prosocial behavior, four groups were compared – Control, MS-F, MS-F, and VMS-F (Fig. 4**A**). Over the HBT days, the percentage of door openings increased and latency to door opening decreased [F(3.853,226.6) = 28.84, $p < 0.0001$; Fig. 4**B-D**]. However, the proportion of pairs that opened the door consistently (classified as openers) was significantly lower in the control group [$\chi^2(1) = 11.00$, $p = 0.0009$; Fig. 4**E**] than all stress groups combined. Moreover, on the last three days of the behavioral paradigm, door opening latencies in the control group were significantly longer compared to each of the three stress groups, reflecting the fewer door openings in the control group [F(3,8) = 31.34, $p < 0.0001$; Fig. 4**F**[.

3.2.2. Microbiome-prosocial-stress interplay

Next, we used 16S rRNA gene sequencing to investigate overall gut microbiome differences between the four groups. Alpha diversity did not significantly differ between the groups; however, comparisons of beta diversity indicated significant differences between the four groups [F (3,92) = 3.013, $p = 0.001$], with no significant interaction between groups and timepoints (before and after HBT; $p = 0.218$). Pairwise post-hoc analysis revealed that the control group differed from all stress groups in its beta diversity (MS-F: $p = 0.012$, MS-T: $p = 0.002$, VMS-F: $p = 0.002$). Interestingly, MS-T also significantly differed from VMS-F ($p = 0.036$). DESeq2 analyses revealed specific taxa that differentiate the microbiome profile of the control group from the other three stress groups (Fig. 5**A-C** and **Table S2**). A Venn diagram highlights the number of differentially abundant bacterial taxa between each treatment and the control that overlap across the three stress groups as well as the unique non-overlapping taxa which differed from the controls (Fig. 5**D**).

The differences in microbiota between the control and the stress groups are maintained when separating the samples by the different sampling times (before and after) and according to helping status (openers and non-openers; see **Fig. S3 and S4**). However, a different pattern emerges when comparing the three stress groups. No clear clustering was apparent between the three stress groups in microbiome profiles following unbiased hclust clustering of the identified significant taxa (pheatmap package in R; see **Fig. S5**). Nevertheless, while the differences among the openers between the stress groups are minimal, there are clear differences between the three groups among the non-openers (Fig. 6**A-C**). A Venn diagram shows the overlapping differentially abundant bacteria, indicating which taxa are uniquely different between each stress group and the other two groups (Fig. 6**D**). MS-T non-openers differed from the other groups with increased relative abundance of *CAG-41, OEMS01*, and *Paramuribaculum intestimale*, while *Akkermansia muciniphila, Bacteroides sartorii, C941, COE1, Muribaculum,* and *Oscillibacter* were relatively decreased. MS-F non-openers differed from the other groups in the relatively decreased taxa *CAG-110, Thomasclavelia clcleatum* (formerly *Clostridium cocleatum*)*, Parabacteroides merdae,* and *UBA2658.* VMS-F non-openers only differed from both groups in the relatively decreased taxon *Roseburia*.

4. Discussion

4.1. Eubacterium and Prosocial Behavior

In the current study, we investigated the relationship between the gut microbiome, prosocial behavior, and stress. Our first experiment demonstrated significant differences in prosocial behavior patterns between individuals classified as 'openers' and 'non-openers' in the HBT.

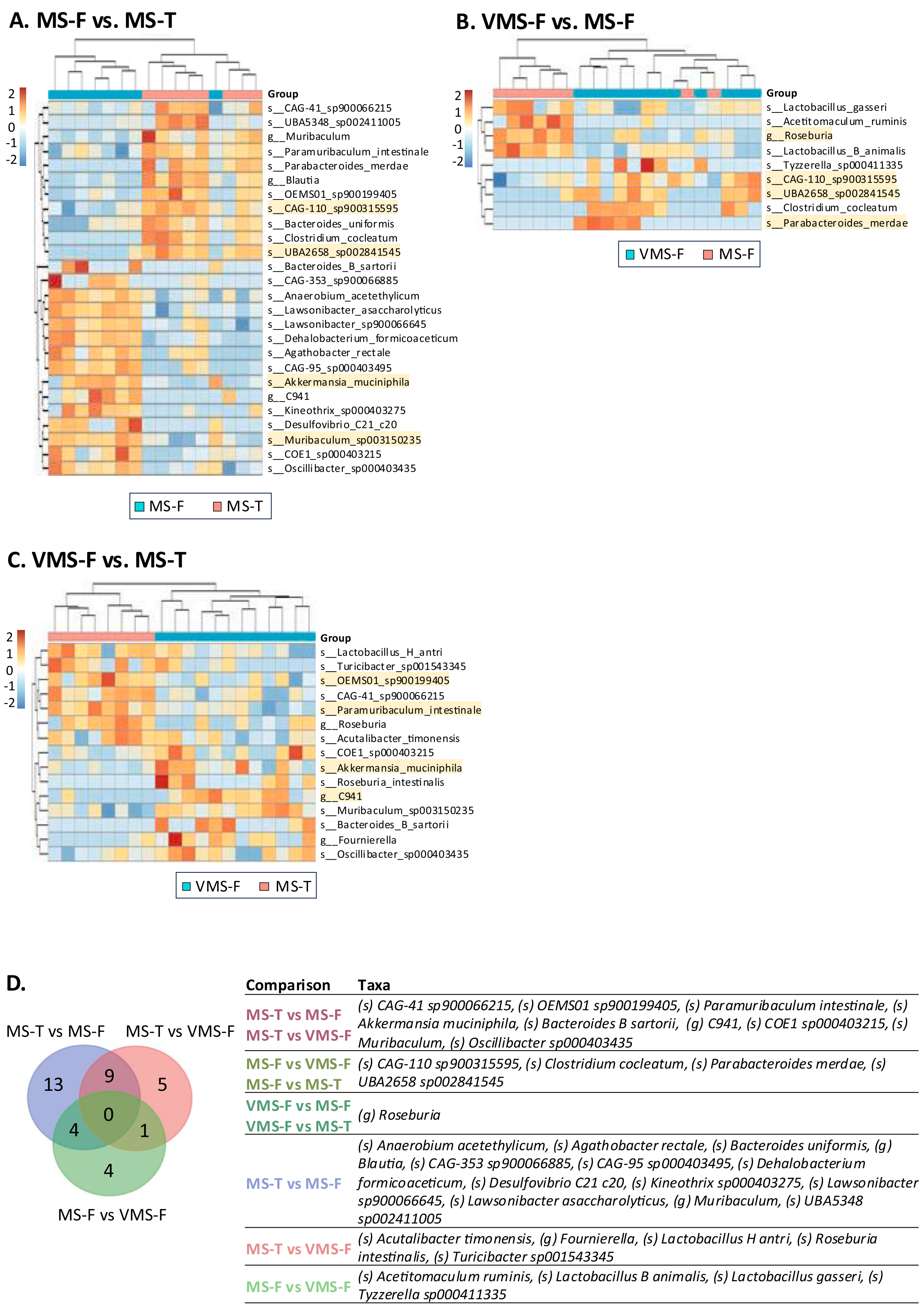
Further investigation into the gut microbiome composition of openers and non-openers revealed significant differences in the abundance of specific taxa. We used two fecal sampling time-points for microbiota profiling. The first sampling aimed to represent innate/baseline differences in the microbiome prior to behavioral testing, whereas the second could reveal microbiota alterations resulting from the HBT. As expected, we observed no baseline differences in microbiome profiles between naïve openers and non-openers before the HBT, which was anticipated given that the rats were raised in the same environment and had not undergone any differential procedures. Nevertheless, after the HBT, we discovered three taxa that varied between openers and non-openers. Thus, the profile of gut microbes appears to be susceptible to the different social experiences of openers and non-openers in the "trapped rat" situation. Thus, the observed differences in the microbiome may represent responses to the HBT paradigm rather than microbiome-driven behavioral changes.

Specifically, *E. ventriosum* and *E. CAG-180*, both members of the genus *Eubacterium*, showed significantly higher relative abundance in openers, while the genus *COE1* demonstrated reduced relative abundance. These findings suggest a potential link between the gut microbiota and prosocial behavior, aligning with emerging evidence indicating the role of the gut microbiome in modulating social behavior (Grimaldi et al., 2018; Ligezka et al., 2021). Notably, the findings regarding *E. CAG-180* were replicated in Experiment 2, where *E. CAG-180* abundances were elevated in the stress groups exhibiting higher prosociality. The genus *Eubacterium* has been associated with social skills in children diagnosed with autism spectrum disorders, with a decreased abundance observed in the autism group compared to the control (Laue et al., 2020; Liu et al., 2019). Furthermore, children with autism who received probiotics and oxytocin treatment showed improved social scores, and this improvement was correlated with the abundance of *Eubacterium* (Kong et al., 2021). Our current findings strengthen this growing body of evidence linking *Eubacterium* species to social behavior and suggest that future studies should further investigate the therapeutic potential of *Eubacterium* species as a next-generation probiotic for addressing social behavior deficits.

There are well-documented sex differences in microbiome composition (Jašarević et al., 2016; Stapleton et al., 2024; Valeri and Endres, 2021). A recent study in humans found that at age two, higher alpha diversity correlated with better social and adaptive skills in boys but worse outcomes in girls (Laue et al., 2022). In contrast, we found no significant sex-based differences in prosocial behavior or gut microbiome composition in naïve rats. This may be due to the absence of strong physiological or environmental challenges, as most prior studies reporting sex differences involved stress (Salberg et al., 2023; Tanelian et al., 2024) or specific dietary interventions (Daly et al., 2022; Shastri et al., 2015). Thus, the HBT may not have been a sufficiently potent manipulation. While social interactions can alter the microbiome (Baniel and Charpentier, 2024; Brown et al., 2024), further work is needed to understand how sex modulates these effects, particularly under stress.

4.2. NAc-Gut-Microbiome Pathway in the Context of Social Behavior

Recent studies suggest a bidirectional relationship between the gut microbiome and brain regions involved in social behavior, such as the nucleus accumbens (NAc) (Arentsen et al., 2015; Dong et al., 2022; Lee et al., 2022; Tillisch et al., 2013). In our study, several genes in the NAc, including *Oxtr*, *Adra2a*, and *Adra1d*, were differentially expressed between openers and non-openers, consistent with prior evidence linking NAc activity and social behavior (Gregory et al., 2009; Güroğlu, 2022; Harbaugh et al., 2007; Knafo-Noam et al., 2018; van der Meulen et al., 2016; Zhao and Gammie, 2018). *Eubacterium* correlated with several of these genes, including *Adra1d*, *Trpc7*, and *ACE*, suggesting a gut-brain interaction. Downregulation of *ACE* in openers fits previous findings on *ACE* inhibitors' beneficial effects on social behavior (Mecawi et al., 2009; Srinivasan et al., 2003; Trieu et al., 2022). Likewise, *Trpc7* upregulation may reflect its role in oxytocin release, with knockout models showing social deficits (Higashida, 2016; Higashida et al., 2018; Jang et al., 2015; Mahmuda et al., 2020; Zhong et al., 2016).



Comparison	Taxa
MS-T vs MS-F MS-T vs VMS-F	*(s) CAG-41 sp900066215, (s) OEMS01 sp900199405, (s) Paramuribaculum intestinale, (s) Akkermansia muciniphila, (s) Bacteroides B sartorii, (g) C941, (s) COE1 sp000403215, (s) Muribaculum, (s) Oscillibacter sp000403435*
MS-F vs VMS-F MS-F vs MS-T	*(s) CAG-110 sp900315595, (s) Clostridium cocleatum, (s) Parabacteroides merdae, (s) UBA2658 sp002841545*
VMS-F vs MS-F VMS-F vs MS-T	*(g) Roseburia*
MS-T vs MS-F	*(s) Anaerobium acetethylicum, (s) Agathobacter rectale, (s) Bacteroides uniformis, (g) Blautia, (s) CAG-353 sp900066885, (s) CAG-95 sp000403495, (s) Dehalobacterium formicoaceticum, (s) Desulfovibrio C21 c20, (s) Kineothrix sp000403275, (s) Lawsonibacter sp900066645, (s) Lawsonibacter asaccharolyticus, (g) Muribaculum, (s) UBA5348 sp002411005*
MS-T vs VMS-F	*(s) Acutalibacter timonensis, (g) Fournierella, (s) Lactobacillus H antri, (s) Roseburia intestinalis, (s) Turicibacter sp001543345*
MS-F vs VMS-F	*(s) Acetitomaculum ruminis, (s) Lactobacillus B animalis, (s) Lactobacillus gasseri, (s) Tyzzerella sp000411335*

Fig. 6. Microbiome Differences between Non-Openers from the Stress Groups. (A-C) Heatmaps of the normalized taxa abundances differ significantly between the non-openers from the stress group (MS-F vs. MS-T (A); VMS-F vs. MS-T (B), and VMS-F vs. MS-T (C). Z scores are represented by the color gradient. The four most significant taxa are marked in yellow. (D) A Venn diagram demonstrates the overlapping and unique significantly differentially abundant taxa in the three comparisons between the non-openers from the stress groups with the control group (n = 8 in MS-F, n = 8 in MS-T, and n = 12 in VMS-F). Abbreviations: MS-F, Maternal Separation Free; MS-T, Maternal Separation Trapped; VMS-F, Vicarious Maternal Separation Free. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We also found that immune-related genes (Itk, Clcf1, Itgb6, H2-Bl, H2-Q7) were differentially expressed and associated with *E. ventriosum* and *E. CAG-180* abundance, indicating potential immune involvement in prosocial behavior (Elmore et al., 2020; García-Cabrerizo et al., 2021; Gomez-Rodriguez et al., 2011; Meecham and Marshall, 2020; Mukherjee et al., 2020; Nelson-Coffey et al., 2017; Smith-Garvin et al., 2009).

4.3. *The Possible Modulating Role of Serotonin*

Given the brain's role in regulating both social behavior and the gut microbiome, we examined whether specific genes might influence *Eubacterium* abundance. RNA-seq analysis revealed correlations between *Eubacterium* and NAc-expressed genes, prompting an *in vitro* follow-up using additives identified in our data (oxytocin, ACE, adrenaline) and known modulators of social behavior (serotonin and dopamine; Gunaydin et al., 2014; Kiser et al., 2012; Y. Liu & Wang, 2003; Moskowitz et al., 2001; Shahrokh et al., 2010). These compounds affect both brain and gut physiology (Boyanova, 2017; Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Dave et al., 2016; Luczynski et al., 2016; Monstein et al., 2004; Ohlsson et al., 2006; Wang et al., 2021; Xie et al., 2022).

Notably, serotonin increased *Eubacterium* levels in male but not female fecal cultures, despite despite the absence of sex differences in behavior or microbiota *in vivo*. This aligns with evidence of sex-specific serotonergic effects (Clarke et al., 2013; Lyte et al., 2022; Poceviciute et al., 2023), although findings have been mixed (Wu et al., 2021b). The discrepancy may stem from controlled *in vitro* conditions, whereas *in vivo* behavior reflects complex systems that may mask subtle sex effects.

Serotonin, primarily synthesized in the gut (Gershon and Tack, 2007; Reigstad et al., 2015; Yano et al., 2015), regulates mood and social interactions (Bernasconi et al., 2015; Crockett et al., 2008; Donaldson et al., 2014; Harmer, 2008; Young and Leyton, 2002). Certain gut microbes can modulate serotonin production (Clarke et al., 2013; Strandwitz, 2018; Yano et al., 2015), supporting a model in which serotonin mediates gut-brain signaling in a sex-dependent manner.

4.4. *Direct and Indirect Stress Increase Prosocial Behavior*

In our second experiment, we examined how ELS affected prosocial behavior and the gut microbiome. As the 'trapped rat' situation in the HBT model is an arousing experience, we investigated the effect of ELS on stress reactivity later in life (Ben-Ami Bartal et al., 2016; Sato et al., 2015). ELS is known to impact later affective and social behavior (Holland et al., 2014; Pechtel and Pizzagalli, 2011). We found that both direct and indirect early stressors enhanced prosocial behavior in the HBT compared to naïve controls, consistent with studies showing that moderate stress promotes social bonding, while reducing stress pharmacologically decreases helping (Ben-Ami Bartal et al., 2016; Muroy et al., 2016). These findings support the idea that moderate stress may enhance prosociality, whereas extremes diminish it. However, maternal separation effects might reflect a general ELS response beyond social behavior, warranting further investigation into stressor-specific effects and mechanisms.

4.5. *Stress-Induced Changes in the Gut Microbiome*

In accordance with the behavioral results, the analysis of the gut microbiome composition revealed significant differences between the control group and the three stress groups, with stress altering beta diversity and increasing taxa primarily from the Bacteroidota phylum. This aligns with reports of ELS-induced increases in Bacteroidota (Hantsoo and Zemel, 2021; Kuti et al., 2020; Park et al., 2021) and changes in beta diversity following stress (Rocca et al., 2019). Moreover, the genus *Prevotella* from the Bacteroidota phylum, was found to be relatively increased in the three stress groups. In previous studies, *Prevotella* was associated with social dominance behavior (Agranyoni et al., 2021), higher brain reactivity to emotional stimuli, and higher connectivity between brain circuitry involved in prosocial behavior (Ben-Ami Bartal et al., 2021; Breton et al., 2022; Gao et al., 2019; Tillisch et al., 2017). Taken together, the results suggest that stress can modulate the gut microbial community in association with social behavior.

Co-housing may also have affected the microbiome via coprophagy, particularly in MS-T free rats co-housed with stressed partners. Such microbial transfer has been shown to alter social behavior (Buffington et al., 2016; Sarkar et al., 2020). Additionally, although VMS-F rats weren't directly stressed, their behavior and microbiota resembled those of stressed groups, highlighting the potential impact of stress contagion and indirect exposure on both behavior and microbial profiles.

4.6. *Between Asociality and Distress: Are all Non-Helpers the Same?*

Among the non-openers, we found that the three stressed groups differed from each other in taxonomic composition, while the three stressed opener groups shared similar microbiota. These findings suggest that the different stressors may induce differences in stressed non-openers' microbiome profiles and the way they experience the 'trapped rat' paradigm. One explanation may be related to the U-shaped relationship between ELS and stress responsivity, suggesting that different levels of stress produce different biological and behavioral phenotypes, including different microbiome changes (Hantsoo and Zemel, 2021; Shakiba et al., 2020). For example, the non-opener MS-T rats were exposed to a stressful situation for the first time during the HBT with a stressed trapped cagemate, while the MS-F and VMS-F were previously exposed to direct and indirect early-life stress. These non-opener MS-T rats also differ from the two other stress groups in 9 taxa. This is in accordance with our findings that the stressed non-openers differ in known stress-related phyla – Bacillota (formerly Firmicutes) and Bacteroidota – suggesting that they were affected differently by the different stressors (Guangorena-Gómez et al., 2022; Pusceddu et al., 2015; Rincel et al., 2019; Zhu et al., 2019).

It is also possible that non-opening reflects different internal states, some rats may lack prosocial drive due to stress, while others may have cognitive or physiological limitations. Previous work supports prosocial motivation in the HBT, showing that rats prioritize helping over food (Blystad et al., 2019) and that social brain regions like the NAc are activated post-task (Ben-Ami Bartal et al., 2021, 2014, 2011; Breton et al., 2022). Further work is needed to explore non-openers' underlying mechanisms.

While the current study provides valuable insights, several limitations must be acknowledged for a comprehensive understanding of the findings. The primary aim of the current experiments was to investigate the relationship between the gut microbiome and prosocial behavior. As such, we examined the gut-brain axis only in the naïve state; the stress manipulation served as an exploratory component to examine the microbiome-prosocial behavior relationship from another perspective. This focused approach creates opportunities for future studies to specifically examine the relationship between stress, prosocial behavior, and the gut-brain axis, incorporating additional relevant measurements. Another potential limitation of our study are that litter and batch effects were not fully controlled and the number of samples analyzed by the RNA-seq was relatively small. Moreover, pair-housing may have led to some microbial exchange between cage-mates, potentially influencing individual microbiome data. Nevertheless, we used approximately two pups per litter per group to maintain microbial diversity, and the statistical comparisons between groups were not compromised by coprophagy, as we paired 'free' and 'trapped' rats as cage-mates.

5. Conclusions

Our study reveals distinct microbiome profiles between prosocial rats (openers) and less prosocial (non-openers) rats, both in naïve conditions and following stress exposure. We found elevated levels of

Eubacterium species (*Eubacterium CAG-180* and *Eubacterium ventriosum*) in opener rats. *E. CAG-180* was also elevated in the three stress groups compared to control rats, matching their higher prosocial performances. These microbial differences were also correlated with several genes and pathways in the NAc, including markers of immune function. Our integrative analyses provide new valuable insights into the relationship between prosocial tendencies and the gut-brain-microbiota axis. These findings strengthen the association between *Eubacterium* species and social behavior, indicating that future studies investigating *Eubacterium*-based therapeutic interventions could be valuable for treating social behavior deficits and stress-related disorders.

- Ethics approval and consent to participate. Not applicable
- Consent for publication. Not applicable
- Availability of data and material. Sequencing data has been uploaded and can be found at EBI under the accession number ERP161080. The RNA data are available at https://osf.io/jg7ce/.
- Competing interests. The authors declare that they have no competing interests.
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- Authors' contributions. R.H., A.W., O.K., and I.B.B. designed the study. R.H. collected the samples, acquired the data, and analyzed the behavioral data. L.M. and S.T. and E.S conducted the microbiome analysis. E.E. and N.S. conducted the *in vitro* phase, including qRT-PCR and analysis. R.H., A.W., O.K., and S.T. interpreted the results. R.H. drafted the manuscript. All authors read the manuscript and approved the final version to be published.

CRediT authorship contribution statement

Reut Hazani: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Aron Weller:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Sondra Turjeman:** Writing – review & editing, Writing – original draft, Formal analysis. **Efrat Sharon:** Formal analysis. **Natalia Saleev:** Formal analysis. **Lelyan Moadi:** Formal analysis. **Evan Elliott:** Writing – review & editing, Supervision. **Inbal Ben-Ami Bartal:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Omry Koren:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2025.06.023.

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