Resilience or susceptibility to traumatic stress: Potential influence of the microbiome

Arax Tanelian [a], Bistra Nankova [a,b], Mariam Miari [c], Roxanna J. Nahvi [a], Esther L. Sabban [a,*]

[a] *Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY, USA*
[b] *Division of Newborn Medicine, Department of Pediatrics, New York Medical College, Valhalla, NY, USA*
[c] *Department of Clinical Sciences in Malmo, Lund University Diabetes Center, Malmo, Sweden*

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ABSTRACT

Exposure to traumatic stress is a major risk factor for development of neuropsychiatric disorders in a sub-population of individuals, while others remain resilient. The mechanisms and contributing factors differentiating between these phenotypes are still unclear. We hypothesize that inter-individual differences in the microbial composition and function contribute to host resilience or susceptibility to stress-induced psychopathologies. The current study aimed to characterize gut microbial community before and after exposure to traumatic stress in an animal model of PTSD. Sprague-Dawley male rats were randomly divided into unstressed controls and experimental group subjected to Single Prolonged Stress (SPS). After 14 days, behavioral analyses were performed using Open Field, Social Interaction and Elevated Plus Maze tests. Based on the anxiety measures, the SPS group was further subdivided into resilient (SPS-R) and susceptible (SPS–S) cohorts. The animals were sacrificed after the last behavioral test and cecum, colon, hippocampus, and medial prefrontal cortex were dissected. Prior to SPS and immediately after Open Field test, fecal samples were collected from each rat for 16S V3–V4 ribosomal DNA sequencing, whereas urine samples were collected before SPS, 90 min into immobilization and on the day of sacrifice to measure epinephrine and norepinephrine levels. Analyses of the fecal microbiota revealed significant differences in microbial communities and in their predictive functionality among the groups before and after SPS stressors. Before SPS, the SPS-S subgroup harbored microbiota with an overall pro-inflammatory phenotype, whereas SPS-R subgroup had microbiota with an overall anti-inflammatory phenotype, with predictive functional pathways enriched in carbohydrate and lipid metabolism and decreased in amino acid metabolism and neurodegenerative diseases. After SPS, the gut microbial communities and their predictive functionality shifted especially in SPS cohorts, with volatility at the genus level correlating inversely with Anxiety Index. In line with the alterations seen in the gut microbiota, the levels of cecal short chain fatty acids were also altered, with SPS-S subgroup having significantly lower levels of acetate, valerate and caproate. The levels of acetate inversely correlated with Anxiety Index. Interestingly, urinary epinephrine and norepinephrine levels were also higher in the SPS-S subgroup at baseline and during stress, indicative of an altered sympathoadrenal stress axis. Finally, shorter colon (marker of intestinal inflammation) and a lower claudin-5 protein expression (marker for increased blood brain barrier permeability) were observed in the SPS-S subgroup. Taken together, our results suggest microbiota is a potential factor in predisposing subjects either to stress susceptibility or resilience. Moreover, SPS triggered significant shifts in the gut microbiota, their metabolites and brain permeability. These findings could lead to new therapeutic directions for PTSD possibly through the controlled manipulation of gut microbiota. It may enable early identification of individuals more likely to develop prolonged anxiogenic symptoms following traumatic stress.

* Corresponding author. Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY, 10595, USA.
E-mail addresses: atanelia@student.touro.edu (A. Tanelian), Bistra_Nankova@nymc.edu (B. Nankova), mariam.miari@med.lu.se (M. Miari), rnahvi@student.nymc.edu (R.J. Nahvi), sabban@nymc.edu (E.L. Sabban).

1. Introduction

Stress-induced psychopathologies such as anxiety, major depressive disorder (MDD) and post-traumatic stress disorder (PTSD) are the most prevalent mental health disorders, affecting more than half a billion people world-wide each year and imposing a significant burden on society (World Health Organization, 2017). However, the responses to a similar traumatic experience are significantly different across individuals, both in humans and animal models (Cathomas et al., 2019). Currently stress is no longer viewed as a risk factor for disease with predetermined adverse outcomes, but rather as a factor inducing different phenotypes in individuals (Bryant et al., 2015).

Over the past decade, research on resilience - "the process of adapting well in the face of adversity" has received increased attention. It has been intensively studied in several kinds of acute and chronic stress models (Cathomas et al., 2019; Franklin et al., 2012; Pfau and Russo, 2015). Yet a better understanding of the contributing factors for individual differences in the behavioral outcomes following a traumatic experience is still lacking.

An important biological factor contributing to variations between individuals and playing a critical role in host stress-responses and stress-resilience is the gut microbiota (Aktipis and Guevara Beltran, 2021; Bear et al., 2021). Colonization of the mammalian gut by the microbiota is an evolutionary-driven process that impacts host physiology and behavior (Molina-Torres et al., 2019; Forsythe and Bienenstock, 2016). The gut-brain axis, a complex multi-organ dynamic signaling system, includes the GI microbiome, immune cells, gut tissue, glands, autonomic nervous system, and the central nervous system, which interact bidirectionally to provide appropriate and coordinated, physiological responses essential for survival (Cryan et al., 2019). Extended disturbances to gut microbial homeostasis may alter immune, neuronal, and hormonal pathways, influencing symptoms of variety of mental health conditions including depression, anxiety, and PTSD (Halverson and Alagiakrishnan, 2020; Rea et al., 2020).

In this regard, emerging evidence has linked gut microbiota with several mood disorders (Huang et al., 2019; Jiang et al., 2015; Zheng et al., 2016, Yang et al., 2019, Wong et al., 2016), which began with the observation of co-morbidity of depression and anxiety in patients with gastrointestinal disorders (Kurina et al., 2001; Lydiard, 2001). Since then, several animal studies have shown that the animal's emotional behavior can be affected by the presence/absence or perturbed composition of the gut microbiota (Lyte et al., 2006; Bercik et al., 2010; Crumeyrolle-Arias et al., 2014). Moreover, both clinical and pre-clinical studies have demonstrated that stress-induced pathologies can be ameliorated using various microbiota-targeted interventions (Bharwani et al., 2017; Ait-Belgnaoui et al., 2014; Liang et al., 2015). Further proof of causality in the connection between gut microbiota and psychiatric disorders comes from fecal-transplantation studies, where fecal transplantation from depressed subjects into healthy animals induced depressive-like behavior in the recipients (Kelly et al., 2016).

Correlational studies have also shown that stress can alter the gut microbiota which may, at least partially, mediate the onset of mood disorders. For instance, stress rodent models of depression exhibit reduced gut microbial richness and diversity, suggesting that the disturbance of microbial composition and/or altered microbial metabolites may contribute to the development of depression (Caspani et al., 2019). Likewise, the gut microbiota of patients with MDD exhibit significant imbalance in the relative abundance of several genera within the main phyla Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria compared to healthy controls (Winter et al., 2018).

The potential role of microbiota in the pathogenesis of PTSD is less examined, despite being proposed to play a key role. Only a few studies focus on the impact of gut microbiome in the development and severity of PTSD (Bersani et al., 2020; Leclercq et al., 2016). One such exploratory study compared the fecal microbiota composition of 18 individuals with PTSD and 12 trauma exposed controls. Three phyla with altered relative abundances were associated with higher PTSD severity (Hemmings et al., 2017). Altered gut-brain functionality was also associated with combat-related PTSD in veterans with cirrhosis (Bajaj et al., 2019).

To further investigate the input of gut bacteria to distinct stress-related behavioral outcomes, we took advantage of the widely used rodent model of PTSD – Single Prolonged Stress (SPS) (Liberzon and Young, 1997; Lisieski et al., 2018a). Two weeks after SPS exposure, only a subset of the animals display anxiety-like behavior (susceptible) (Le Dorze and Gisquet-Verrier, 2016; Serova et al., 2019a), while the others behave like unstressed controls (resilient). Therefore, SPS is an appropriate model to investigate differences in the microbiome before and after the traumatic stress in animals that are resilient and susceptible to SPS triggered anxiety.

Given that significant individual-to-individual differences in taxonomic composition of the commensal microbiota have been reported in humans and in controlled populations of inbred laboratory animals (Turnbaugh et al., 2009; Ley et al., 2006; Hoy et al., 2015), we hypothesized that existing individual differences in the gut microbiota composition and functionality might predispose the host to resilience or susceptibility to traumatic stress-induced behavioral impairments. In this study we aimed to:

(1) Determine whether pre-existing differences in gut microbiota composition and functionality are associated with SPS-triggered stress resilience or vulnerability; and
(2) Determine how SPS affects gut microbial composition, functionality, and the gut-brain axis in resilient and susceptible animals.

2. Material and methods

2.1. Animals

All animal experiments complied with ARRIVE guidelines and with NIH Guide for Care and Use of Laboratory animals. They were approved by the New York Medical College's Institutional Animal Care and Use Committee (IACUC).

Sprague-Dawley outbred male rats (150–160g) were purchased from Charles River Laboratories (Wilmington, MA, USA). Upon arrival, animals were housed four per cage, and were maintained under a 12-h light/dark cycle, at $23 \pm 1\,^{\circ}$C. Food and water were provided *ad libitum*.

2.2. Experimental timeline

The experimental timeline is shown in Fig. 1. After 14 days of accommodation to the animal facility, rats were randomly assigned into either unstressed control (**n = 10**) or experimental group (**n = 14**). The experimental group was subjected to SPS, while the control group was briefly handled. After SPS, animals from both groups were housed 2 per cage. The experimental group was left undisturbed without bedding changes for 7 days, to consolidate the experience of traumatic stress, after which they were kept with normal bedding changes for the remainder of the experiment. Two weeks after SPS (day 31) all animals were exposed to a battery of behavioral tests, performed in the following order: Open Field (OF), Social Interaction (SI), and Elevated Plus Maze (EPM) test.

Stool and urine samples were collected at the times indicated in Fig. 1. All behavioral tests and stool samples collection were performed between 10 a.m. and 3 p.m. to minimize circadian influences on the microbiome. The animals were weighed after SPS and after each behavioral test. One day after the last behavioral test, the animals were sacrificed by decapitation and different organs were collected.

2.3. Single Prolonged Stress (SPS)

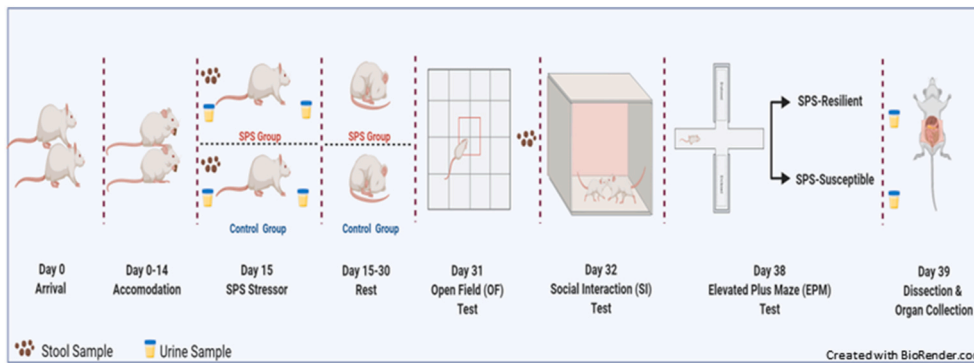SPS, a widely used model for PTSD, elicits a strong stress response

Fig. 1. *Experimental design.* The animals were allowed to accommodate to the animal facility for 14 days upon arrival. On day 15, they were randomly assigned into unstressed control or exposed to SPS group. The SPS group was left undisturbed for 7 days, after which they were kept with normal bedding changes for the remainder of the experiment. On day 31 the controls and the SPS group were exposed to battery of behavioral tests in the following order: Open Field (OF), Social Interaction (SI), and Elevated Plus Maze (EPM). On day 39, the animals were sacrificed by decapitation and different organs were collected. Animals were divided into SPS-R and SPS-S subgroups based on their performance on OF and EPM tests. Stool samples were collected before SPS and immediately after OF test, and urine samples were collected before SPS, 90 min into immobilization and on the day of sacrifice.

through psychological, physiological, and pharmacological pathways, inducing neurobiological and neuro-immune impairments (Lisieski et al., 2018b, Liberzon and Young, 1997). A slightly modified version of SPS was performed as previously described (Serova et al., 2019a). Briefly, the animals were restrained by taping their limbs with surgical tape to a custom-made metal board which also restricted the motion of their heads. Immediately after 2 h of immobilization the animals were subjected to 20 min forced swim in a plexiglass cylinder (50 cm height, 24 cm diameter; Stoelting, Wood Dale, IL, USA) filled two-thirds with 24 °C fresh water. Then, they were dried and allowed to recuperate for 15 min, after which they were exposed to ether in a glass desiccator chamber until loss of consciousness.

2.4. Behavioral tests

Behavioral tests were administered in order of least to most stressful to reduce possible carryover effects from prior behavioral tests. Animals were tested on Open Filed (OF), where time and number of entries into the center of the OF arena were calculated, on Social Interaction (SI), where duration and number of interactions with a juvenile rat were assessed, and on Elevated Plus Maze (EPM), where duration and entries into open arm (OA) and closed arm (CA) were evaluated. All tests were performed in a room with dim light, videotaped with a ceiling camera and were analyzed by trained individuals blinded to the groups (**Supp. File).**

2.5. Tissue collection

Brains were dissected using a brain matrix. For ventral hippocampus (vHipp) sections, −4.80 mm to −5.20 mm to bregma were dissected and for medial prefrontal cortex (mPFC) sections, 1.5 mm to −3.7 mm to bregma were isolated, flash frozen in liquid nitrogen and stored at −80 °C until further use. The colon was also isolated from each rat, and one cm from the cecum and one cm from the distal end were removed before measuring the tissue's length.

2.6. Fecal microbiota sequencing

To determine the microbiome profile of the cohorts, fecal samples were collected aseptically from individual rats at indicated time points (Fig. 1) and stored at −80 °C until further use. Prior to SPS, stool pellets were collected by placing each animal in a sterile cage for up to 15min to defecate voluntarily. Upon defecation, the pellets were collected in sterile tubes using sterile forceps, and immediately placed on dry ice. Post SPS, stool pellets were collected using sterile forceps while weighing the animals. Total DNA was extracted from each stool sample using DNeasy PowerSoil Pro Kit (Qiagen, cat. # 47014) per

manufacturer's protocol. Extracted DNA was subjected to 16S V3–V4 rDNA sequencing and analysis at Psomagen (Rockville, MD) (**Supp. File**). The 16S sequencing data are deposited to NCBI SRA, accession # PRJNA819002.

2.7. Cecum and cecal SCFA quantification

Ceca were isolated, weighed and snaped frozen in liquid nitrogen and stored at −80 °C until further use. The cecal weight was normalized to body weight measured on the day of dissection. Cecal samples (n = 5/ group) were sent for SCFA analysis (Gnotobiotics, Microbiology, and Metagenomics Center, Boston, MA, US) (**Supp. File**).

2.8. Urine collection and epinephrine/norepinephrine analysis

Urine samples were collected from each rat before SPS (baseline, rats were placed on pads for 20 min and were left to urinate voluntarily), 90 min into immobilization (disposable dishes were placed under each rat) and on the day of dissection (disposable dishes were used while weighing the animals before sacrifice) to measure catecholamine levels. The samples were acidified immediately by addition of an equal volume of 0.01 M HCl and stored at −80 °C for further analysis. Urine epinephrine and norepinephrine levels were quantified using commercially available competitive enzyme immunoassay kit (Rocky Mountain Diagnostics, Colorado Springs, CO) and normalized to urinary creatinine concentrations in the same samples (DetectX Urinary Creatinine Kit, Arbor Assays, Ann Arbor, MI), as described (LaGamma et al., 2021).

2.9. Western blot

Individual samples from vHipp and mPFC were homogenized in RIPA buffer. Protein concentration was determined by DC Protein Assay (Bio-Rad, Hercules, CA) with Bio-Tek plate reader, and 50 μg of total protein were separated on 4–10% gel and transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5% milk for 1h at room temperature and incubated with primary antibody overnight at 4 °C. Three different primary antibodies were used: Anti-Claudin-5 monoclonal antibody (1:500 dilution, Invitrogen Cat # 4C3C2), anti-Occludin monoclonal antibody (1:500 dilution, Invitrogen Cat # OC-3F10) and anti-GAPDH antibody (1:10000, Cell Signaling, Cat # 14C10). GAPDH was used as an internal control. After incubation with secondary antibody (IRDye 800CW) the bands were visualized using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NB) and analyzed using ImageJ.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software. Data were assessed for normality using the Shapiro-Wilk test and for equality of variances using Brown-Forsythe and Bartlett's tests. Comparison of more than two groups was performed by one-way ANOVA followed by Tukey's multiple comparison test for Gaussian distributions, whereas Kruskal-Wallis test followed by Dunn's multiple comparison test was used for non-Gaussian distributions. For comparing group means from different time points, two-way ANOVA, repeated measures, or mixed effect model were used, with post-hoc Šídák's and/or Tukey's multiple comparisons test. For comparing two groups student's *t*-test was used. Pearson's correlation coefficient was used to assess correlation. For further analysis R version 4.1.2 was used. Microbiome data were centered log-ratio (CLR) transformed, using compositions library (Gloor et al., 2017, McLaren et al., 2019). The principal component analysis for beta diversity was performed in R using Aitchison distance as a distance matrix. For metagenomic function prediction, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to infer the KEGG pathways. An α level of less than 0.05 (two-tailed) was set for significance. To correct for multiple testing, the Benjamini-Hochberg (BH) post-hoc test was used with a q-value of 0.05 as a cut-off to analyze microbiome data. A single value/group $\geq$ 2SD away from the mean was removed from microbiome analysis. Data are expressed as mean $\pm$ SEM.

3. Results

3.1. Identification of animals resilient or susceptible to traumatic stress

To select animals with SPS-Susceptible and SPS-Resilient phenotypes we performed the following behavioral tests:

3.1.1. Open Field (OF) test

The OF provides an initial screen for anxiety-related behaviors (Prut and Belzung, 2003). The analysis revealed that the SPS group spent significantly less time in the center of the arena (t = 2.415, df = 22, p = 0.0245) (Fig. 2A) and had significantly fewer number of entries into the center (t = 2.549, df = 22, p = 0.0183) (Fig. 2B) compared to the controls.

We then subdivided the SPS group into SPS-Susceptible (SPS–S) and SPS-Resilient (SPS-R) subgroups. Animals with duration and number of entries into the center of arena 2 SD below the mean of the controls were separated and assigned to the SPS-S subgroup (Alves-dos-Santos et al., 2020). The remaining animals were assigned to the SPS-R subgroup. Using one-way ANOVA, we found significant differences among the groups in the time spent (F $_{(2, 21)}$ = 10.4, p = 0.0007) and in the number of entries into the center of the OF arena (H (3) = 12.44, p = 0.0020). Compared to the controls and SPS-R subgroup, animals in the SPS-S subgroup spent significantly less time (p = 0.0006, p = 0.0062 respectively) (Fig. 2C) and had significantly fewer number of entries into the center of the arena (p = 0.0019, p = 0.0185 respectively) (Fig. 2D).

3.1.2. Elevated Plus Maze (EPM) test

The EPM is used to assess anxiety-like or avoidance behavior in rodents (Walf and Frye, 2007). One-way ANOVA (F $_{(2,21)}$ = 5.523, p =
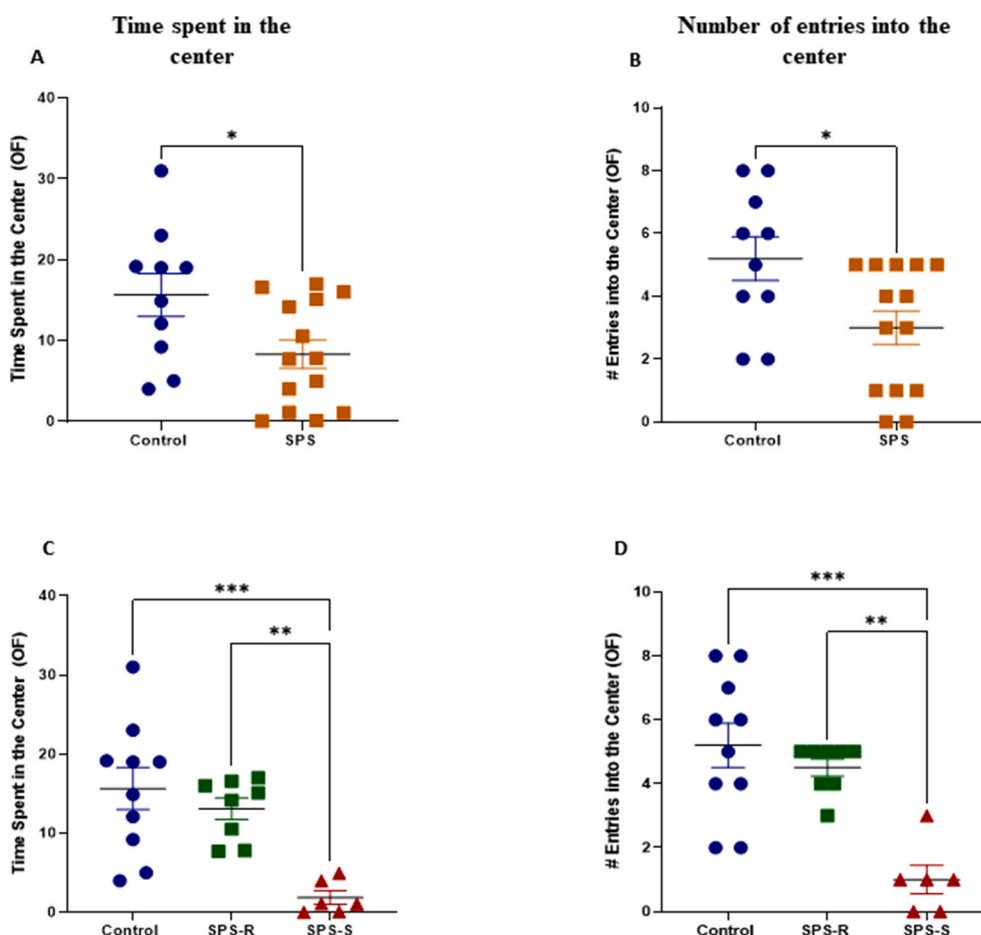

Fig. 2. *Effect of SPS on anxiety-like behavior measured by Open Filed (OF).* Fourteen days after SPS, the animals were tested for: (**A**) Time spent in the center of arena, and (**B**) Number of entries into the center of the arena. Unpaired *t*-test was performed for comparison of the means. After being subdivided into SPS-R and SPS-S subgroups: (**C**) Time spent in the center of and (**D**) Number of entries into the center by SPS subgroups. Number of entries into the center by SPS subgroups data didn't pass the normality test and were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparisons test. Time spent in the center by SPS subgroups data passed the normality test and were analyzed using one way-ANOVA, followed by Tukey's multiple comparisons test. Each dot represents value for an individual animal. Blue-Controls, green-SPS-R, red-SPS-S. All data are expressed as means $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

0.0118) revealed that, the SPS stressors significantly decreased the time spent in the open arms in both SPS-S (p = 0.0184) and SPS-R (p = 0.0474) subgroups relative to the controls (Fig. S1).

Because both OF and EPM tests are putative measures of anxiety and were performed days apart, we continued the analyses with the animals that demonstrated anxiety-like behavior on both tests as measured by the time spent in the center of OF and on the open arms of EPM (Table S1). As a result, the final animal grouping came down to: Controls (n = 7), SPS-R subgroup (n = 6) and SPS-S subgroup (n = 5).

Then, we re-analyzed the different measures on EPM with this grouping. SPS stressors significantly decreased the time spent (F $_{(2,15)}$ = 21.40, p=<0.0001) in the open arm in both SPS-S (p=<0.0001) and SPS-R (p = 0.0079) subgroups relative to the unstressed controls, however, the duration spent by the SPS-S subgroup in the open arm was even lower relative to the SPS-R subgroup (p = 0.0216) (Fig. 3A). The number of entries into the open arms were also different among the groups (F $_{(2,15)}$ = 8.954, p = 0.0028), with SPS-S subgroup having significantly less number of entries relative to the controls (p = 0.0044) and SPS-R subgroup (p = 0.0063) (Fig. 3B). Finally, the overall Anxiety Index (F $_{(2,15)}$ = 13.63, p = 0.0004), which takes into consideration different measurements analyzed on EPM (Cohen and Zohar, 2004), was higher in SPS-S subgroup relative to the controls (p = 0.0003) and the SPS-R subgroup (p = 0.0117) (Fig. 3C).

3.1.3. Social Interaction (SI) test

The SI test is used to assess active interaction of a test animal with a novel juvenile rat (Varlinskaya and Spear, 2008). There were significant differences among the groups in the time spent interacting (F $_{(2,15)}$ = 3.845, p = 0.044). Animals in the SPS-S subgroup spent significantly less time interacting with the juvenile animal compared to the controls (p = 0.0477) (Fig. 4A). When the number of approaches initiated by the test rats to the juvenile rat were analyzed, one-way ANOVA approached significance (F $_{(2,15)}$ = 3.422, p = 0.059), and Tukey's multiple comparisons test revealed significantly fewer number of approaches initiated by the SPS-S subgroup relative to the controls (p = 0.0484) (Fig. 4B).

3.2. Differences in gut microbial composition/functionality in SPS-R and SPS-S subgroups before and after exposure to SPS

Given that the animals exposed to SPS stressors displayed different behavioral outcomes on the behavioral tests, we first examined whether there are any differences in their gut microbial composition that might predispose them to SPS-susceptibility or resilience; and secondly, determined how SPS stressors affect the gut microbial composition in each subgroup. The 16S V3–V4 rDNA sequencing of fecal samples collected before exposure to SPS and immediately after OF test revealed differences in the microbial communities in SPS-S and SPS-R subgroups at different taxonomic levels.

3.2.1. Alpha, beta diversity, and volatility

Alpha diversity is used to assess differences in within-subjects diversity. Although repeated measures ANOVA did not show any significant differences in group or time effect, yet multiple comparisons test revealed pre-existing differences among the groups, with SPS-R subgroup having significantly lower alpha diversity prior to SPS relative to SPS-S subgroup (p = 0.0348) as measured by Inverse Simpson index. No differences in alpha diversity were seen after SPS (Fig. 5A).

Beta diversity provides a measure of similarity or dissimilarity of the whole microbial community between samples. Using Aitchison distance matrix (Aitchison et al., 2000), as a measure of beta diversity, PCA plot showed a clear separation between the SPS subgroups before SPS (Fig. 5B). No differences in beta diversity were seen after SPS (Fig. 5C).

Next, we quantified the degree of compositional change of the gut microbial community, defined as volatility, before and after SPS, by calculating the intra-individual Aitchison distance between the genus-level clr-transformed abundances (Bastiaanssen et al., 2021). Although one-way ANOVA did not show any significant differences among the groups (Fig. 5D), yet volatility correlated significantly with the different parameters tested on the EPM test. For instance, volatility inversely correlated with Anxiety Index (r = −0.62, p = 0.0079) (Fig. 5E) and the percent duration in CA (r = −0.58, p = 0.015) (Fig. 5F) and positively with percent duration in OA (r = 0.58, p = 0.015) (Fig. 5G).

3.2.2. Differential abundance analysis at genus level

Microbial analysis at higher taxonomic levels before and after SPS can be found in (Figs. S2–S4).

Analysis at the genus level revealed significant differences among the groups: three differences were found only before SPS, two differences only after SPS, and seven differences were seen both before and after SPS.

Before SPS, SPS-R subgroup had significantly higher abundance of Lactobacillus relative to SPS-S subgroup (p = 0.0086) and the controls (p = 0.0335), with two-way ANOVA showing significant over-time differences (F $_{(2,25)}$ = 4.962, p = 0.0153) (Fig. 6A). The abundance of Lactobacillus also inversely and significantly correlated with Anxiety
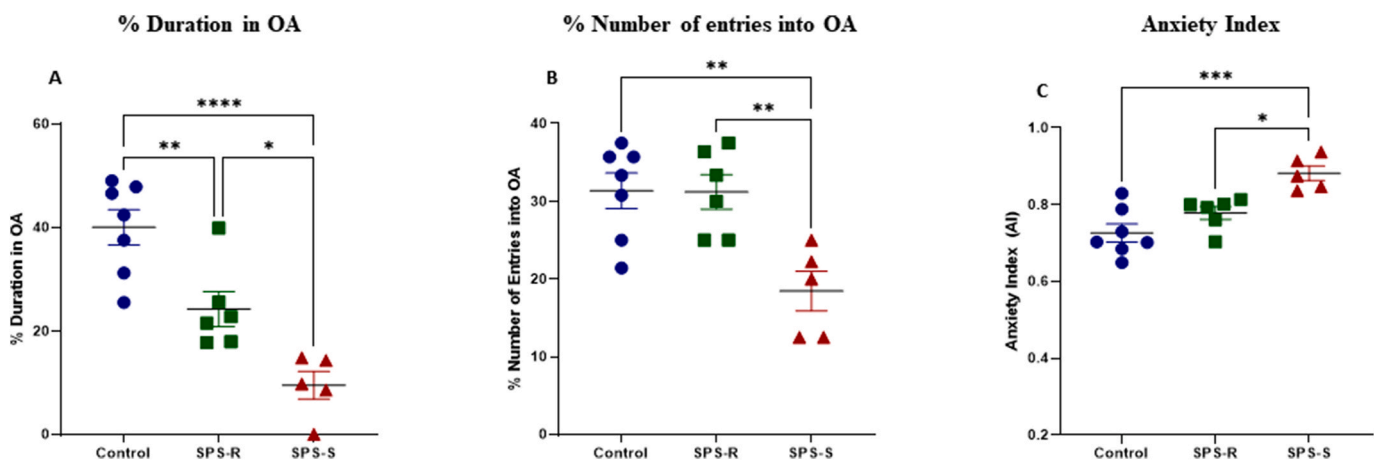

Fig. 3. *Effect of SPS on anxiety-like behavior measured by Elevated Plus Maze (EPM)* Animals were tested on the EPM 23 days after SPS. (**A**) Percent duration in open arm (OA), (**B**) Percent number of entries into the OA, (**C**) Anxiety Index. The EPM data passed the normality test and were analyzed using one way-ANOVA, followed by Tukey's multiple comparisons test. Each dot represents value for an individual animal. Blue-Controls, green-SPS-R, red-SPS-S. All data are expressed as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
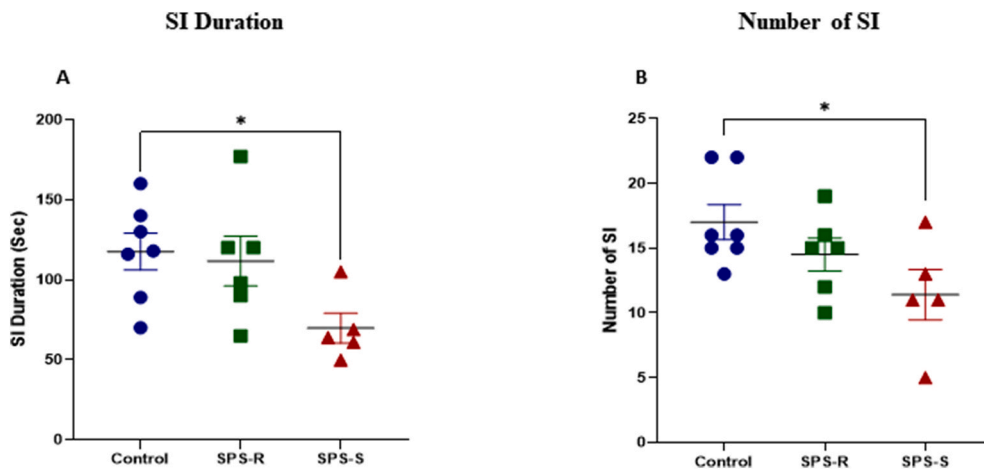
Fig. 4. *Effect of SPS on social impairment measured by Social Interaction (SI) Test.* Social interaction test was performed 15 days after SPS. (**A**) Duration engaged in social interaction and (**B**) number of approaches/interactions initiated by the test rat. The SI data passed the normality test and were analyzed using one way-ANOVA, followed by Tukey's multiple comparisons test. Each dot represents value for an individual animal. Blue-Controls, green-SPS-R, red-SPS-S. All data are expressed as means ± SEM. *p < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Index in the SPS subgroups (r = −0.8944. p = 0.0005) (Fig. 6B). Similarly, the relative abundance of genus Vampirovibrio was significantly higher in SPS-R subgroup compared to the SPS-S subgroup (p = 0.0025) and the controls (p = 0.0294), with significant differences observed over-time (F $_{(2,25)}$ = 3.812, p = 0.0359) (Fig. 6C). On the other hand, genus Lachnospiracea_Incertae_Sedis was significantly higher in SPS-S subgroup relative to the SPS-R (p = 0.0188) (Fig. 6D) and correlated positively with Anxiety Index (r = 0.6341, p = 0.049) (Fig. 6E).

Among the genera which showed significant differences only after SPS was the genus Coprobacillus. Coprobacillus revealed significant group (F $_{(1,23)}$ = 9.014, p = 0.0064) and over-time differences (F $_{(2,23)}$ = 6.349, p = 0.0064). After SPS, its abundance increased in SPS-S subgroup (p = 0.0204) and was significantly higher than the controls (p = 0.0012). Coprobacillus abundance also trended towards increase in SPS-R subgroup relative to the controls (p = 0.0514) (Fig. 6F). Similarly, genus Anaeroplasma was significantly higher in SPS-S subgroup relative to the controls (p = 0.0059) and showed significant over time differences (F $_{(2,25)}$ = 5.409, p = 0.0112) (Fig. 6G).

When analyzing differences in the genera seen both before and after SPS, group differences in the genus Bacteroides approached significance (F $_{(1,26)}$ = 4.171, p = 0.0514), with SPS-R subgroup having significantly higher abundance relative to the SPS-S subgroup (p = 0.0359) and the controls (p = 0.0337) before SPS. The relative abundance of Bacteroides also correlated inversely and significantly with Anxiety Index in the SPS-subgroups (r = −0.7461, p = 0.0132). After SPS, its abundance trended towards increase in SPS-S subgroup (p = 0.066) (Fig. 6H and I).

Similarly, genus Barnesiella showed significant group differences (F $_{(1,25)}$ = 6.922, p = 0.0144), along with significant interaction between group and time (F $_{(2, 25)}$ = 13.89, p = 0.004). Before SPS, its relative abundance was significantly lower in SPS-S subgroup relative to SPS-R (p = 0.0001) and the controls (p = 0.0144) (Fig. 6J) and correlated inversely with Anxiety Index (r = −0.6381, p = 0.0471) in SPS-subgroups (Fig. 6K). However, after SPS, its relative abundance decreased significantly in SPS-R subgroup (p < 0.0001) and increased in SPS-S subgroup (p = 0.0679). As a result, it was significantly higher in SPS-S subgroup relative to SPS-R subgroup (p = 0.0409).

Next, genus Asaccharobacter showed significant group (F $_{(2, 24)}$ = 4.805, p = 0.0176) and over time (F $_{(2,24)}$ = 5.187, p = 0.032) differences. Before SPS, its relative abundance was significantly lower in SPS-R subgroup relative to the SPS-S subgroup (p = 0.0206) and the controls (p = 0.001). However, after SPS, its abundance increased significantly in SPS-R subgroup (p = 0.0046) (Fig. 6L).

Another genus which showed close to significance over-time differences (F $_{(2,24)}$ = 3.392, p = 0.0504) and significant group differences (F $_{(1,24)}$ = 7.838, p = 0.0099) and interaction (F $_{(2,24)}$ = 7.937, p = 0.046) was the genus Butyricicoccus. Before SPS, its abundance was significantly higher in SPS-S subgroup relative to SPS-R (p = 0.0047) and the

controls (p = 0.0134). However, after SPS, the abundance of Butyricicoccus increased significantly in SPS-R subgroup (p = 0.0003) and was significantly higher than the controls (p = 0.0247) (Fig. 6M).

Significant group differences (F $_{(2, 25)}$ = 4.671, p = 0.0189) were also seen with genus Mucispirillum. Before SPS, its relative abundance trended towards higher abundance in SPS-S subgroup relative to the controls (p = 0.0611). However, after SPS, its abundance was significantly higher in SPS-R subgroup relative to the controls (p = 0.005) (Fig. 6N).

Finally, both genus Clostridium IV (F $_{(2,26)}$ = 8.196, p = 0.017) and genus Streptococcus (F $_{(2,24)}$ = 3.710, p = 0.00394) showed significant over-time differences. Before SPS, SPS-S had significantly higher abundance of Clostridium IV relative to SPS-R (p = 0.0108), which remained higher even after SPS (p = 0.0396) (Fig. 6O). As for the genus Streptococcus, SPS-S subgroup had significantly higher abundance relative to the controls (p = 0.0304) before SPS. After SPS its abundance trended towards increase in SPS-R (p = 0.0699) relative to the controls (Fig. 6P). Genera which approached significance can be found in (Fig. S5). Overall, before SPS, SPS-R subgroup harbored microbiota with an anti-inflammatory phenotype, whereas the SPS-S subgroup harbored microbiota with a pro-inflammatory phenotype. After SPS, most of the abundances switched between the subgroups.

Since the link between microbial taxonomic composition and metabolic response is not direct (Moya and Ferrer, 2016), we evaluated the predictive functional profiles of gut microbiota before and after SPS (Fig. 7, Fig. S6). Before SPS, the significant differences in the functional profiles of SPS-R and SPS-S subgroups belonged to cellular processes, metabolism, genetic and environmental information processing, and human diseases. In general, SPS-R subgroup had lower amino acid metabolism, neurodegenerative and cancer pathways, but higher carbohydrate metabolism, Xenobiotics biodegradation and metabolism, and genetic information pathways relative to the SPS-S subgroup and the controls (Fig. 7A). After SPS, again, the significantly different pathways belonged to cellular processes, human diseases, metabolism, and genetic information processing. Interestingly after SPS, pathways in carbohydrate, glycan and lipid metabolism were higher in SPS-S subgroup relative to the SPS-R and the controls (Fig. 7B). For more details see (Supp. file).

3.3. Urinary epinephrine and norepinephrine levels are different among the groups before SPS

The gut microbiota can regulate the output of catecholamines (Xiang et al., 2021) and the autonomic nervous system activity can affect the microbiome indirectly by modulating the intestinal environment, and directly, by host signaling molecules, including catecholamines (Osadchiy et al., 2019). Since we observed clear differences in the gut
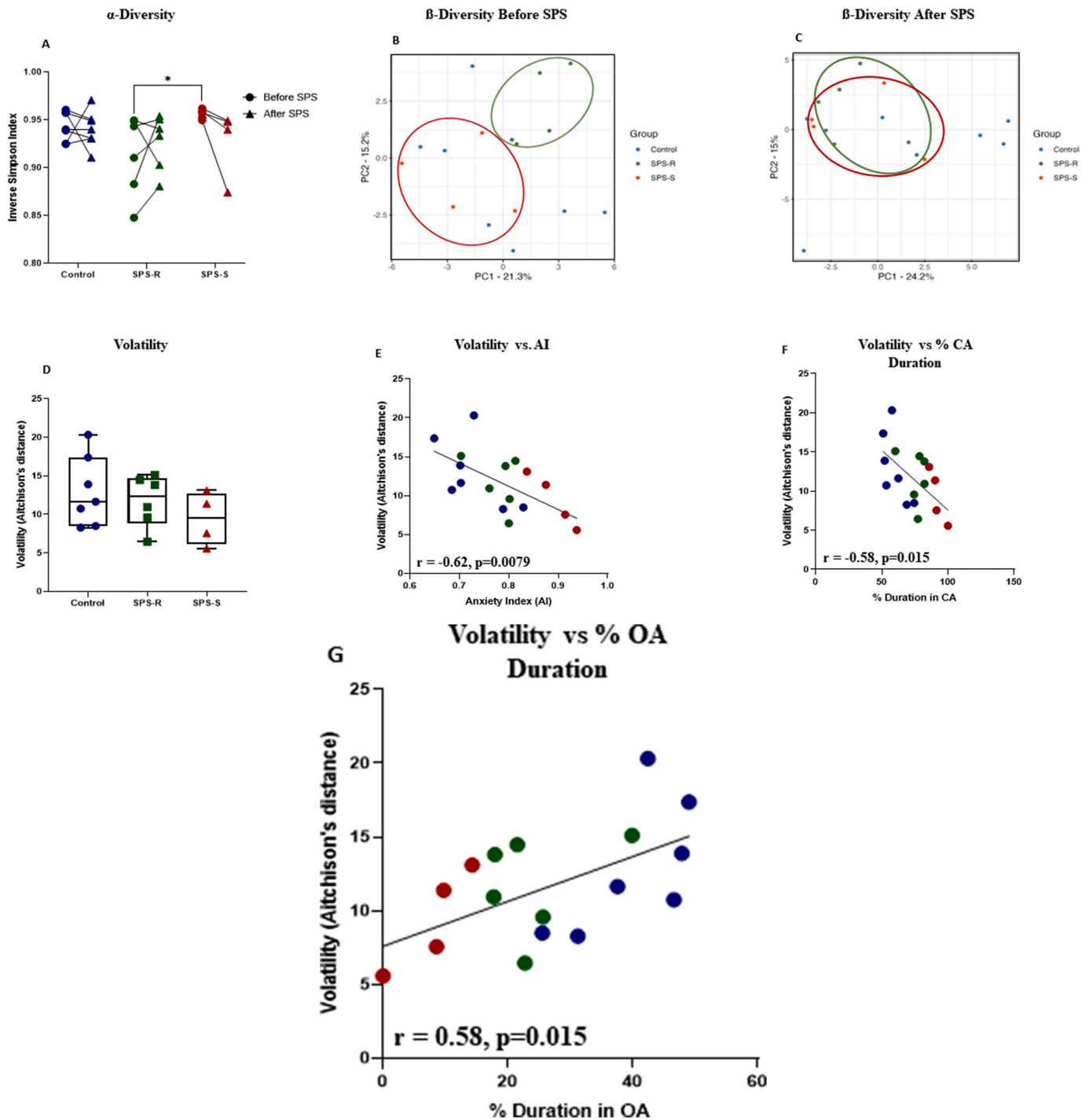
Fig. 5. *Differences in gut microbial alpha and beta diversities among the groups.* The fecal 16S sequencing was used to determine the microbial composition of each group before and after SPS. (**A**) Alpha diversity measured by Inverse Simpson Index, (**B**) PCA plot of Beta diversity before SPS measured by Aitchison distance, (**C**) PCA plot of Beta diversity after SPS measured by Aitchison distance, (**D**) Differences in volatility among the groups, (**E**) Correlation between volatility and Anxiety Index, (**F**) Correlation between volatility and % time spent in the closed arms of EPM, (**G**) Correlation between volatility and % time spent in the open arms of EPM. Inverse Simpson before and after SPS was analyzed by Two-ways ANOVA, mixed effects, followed by Šídák's and/or Tukey's multiple comparisons test. FDR was used to correct between-tests p values. For PCA plots, data points were projected into the space spanned by the first two principal components. Correlations were performed using Pearson's correlation. Blue-Controls, Green-SPS-R, Red-SPS-S. All data are expressed as means ± SEM. *p < 0.05. Values, 2 SD away from the mean were excluded from analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

microbial communities between the subgroups, we next evaluated the dynamics in epinephrine and norepinephrine levels in urine samples collected immediately before SPS, 90 min into immobilization and on the day of dissection. The analyses revealed baseline epinephrine level differences among the groups (F$_{(2,11)}$ = 4.89, p = 0.030). The SPS-S subgroup had significantly higher levels of basal urinary epinephrine

relative to the controls (p = 0.046) and a trend towards increased levels relative to the SPS-R subgroup (p = 0.057). Ninety minutes into immobilization, the urinary epinephrine levels increased significantly in both experimental subgroups (SPS-R, p = 0.0013, SPS-S, p = 0.0006), however the increase was greater in the SPS-S subgroup relative to the SPS-R subgroup (p = 0.0387). By the day of dissection, the levels of
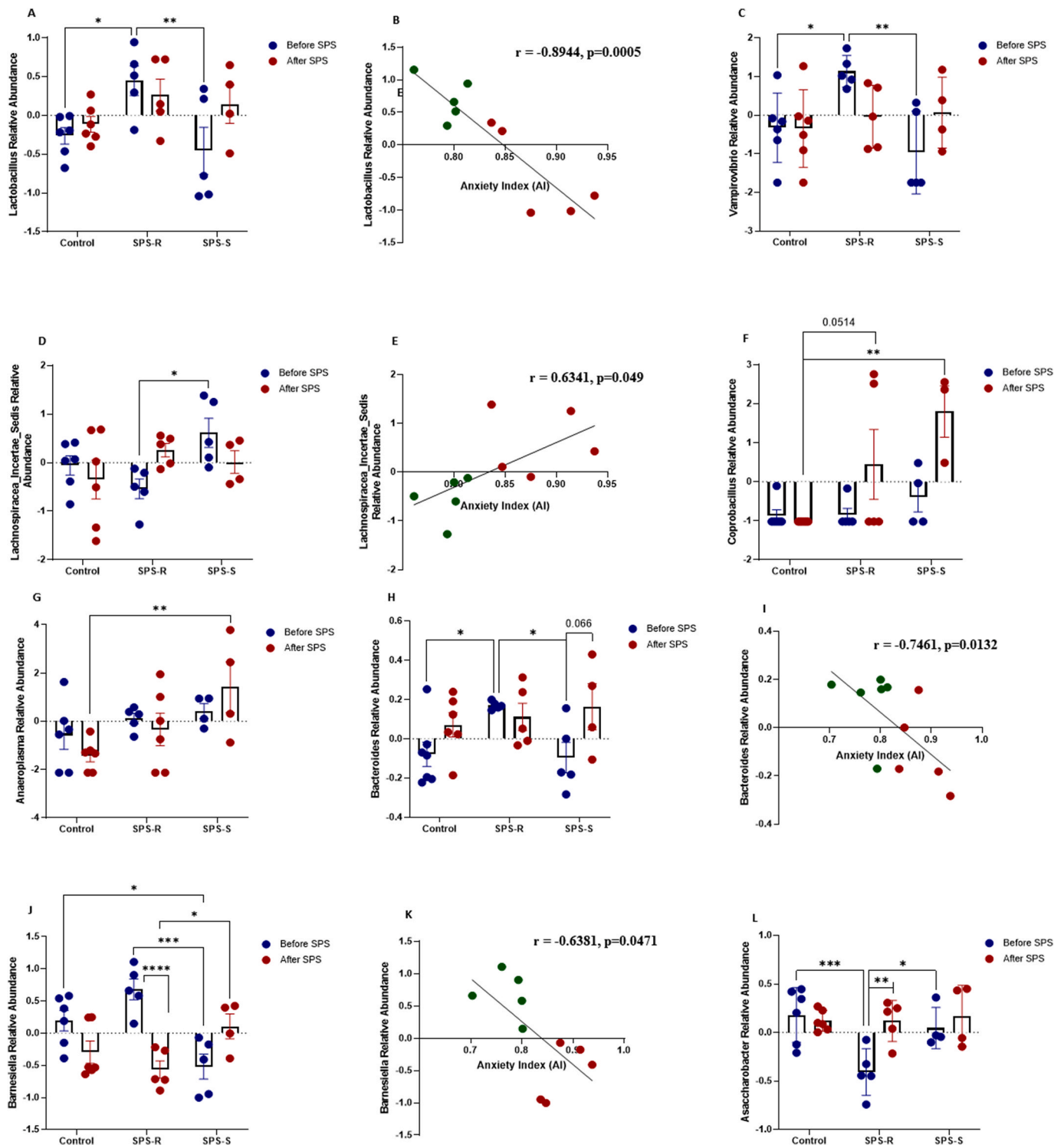
Fig. 6. *Differences in gut microbial communities before and after SPS.* The 16S sequencing was used to determine the microbial composition of each group at genus levels. (**A**) Relative abundances of Lactobacillus, (**B**) Correlation between relative abundance of Lactobacillus and Anxiety Index (AI) before SPS, (**C**) Relative abundance of Vampirovibrio, (**D**) Relative abundance of Lachnospiraceae_Incertae_Sedis, (**E**) Correlation between relative abundance of Lachnospiraceae_Incertae_Sedis and Anxiety Index (AI) before SPS, (**F**) Relative abundance of Coprobacillus, (**G**) Relative abundance of Anaeroplasma, (**H**) Relative abundance of Bacteroides, (**I**) Correlation between relative abundance of Bacteroides and Anxiety Index (AI) before SPS, (**J**) Relative abundance of Barnesiella, (**K**) Correlation between relative abundance of Barnesiella and Anxiety Index (AI) before SPS, (**L**) Relative abundance of Butyricicoccus, (**M**) Relative abundance of Asaccharobacter, (**N**) Relative abundance of Mucispirillum, (**O**) Relative abundance of Clostridium IV, (**P**) Relative abundance of Streptococcus. All relative abundances are clr-transformed. Data were analyzed by Two-way ANOVA followed by Šídák's and Tukey's multiple comparisons. FDR was used to correct between-tests p value. Correlation between relative abundances and AI was performed by Pearson's correlation. Blue-Controls, green-SPS-R, red-SPS-S. All data are expressed as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
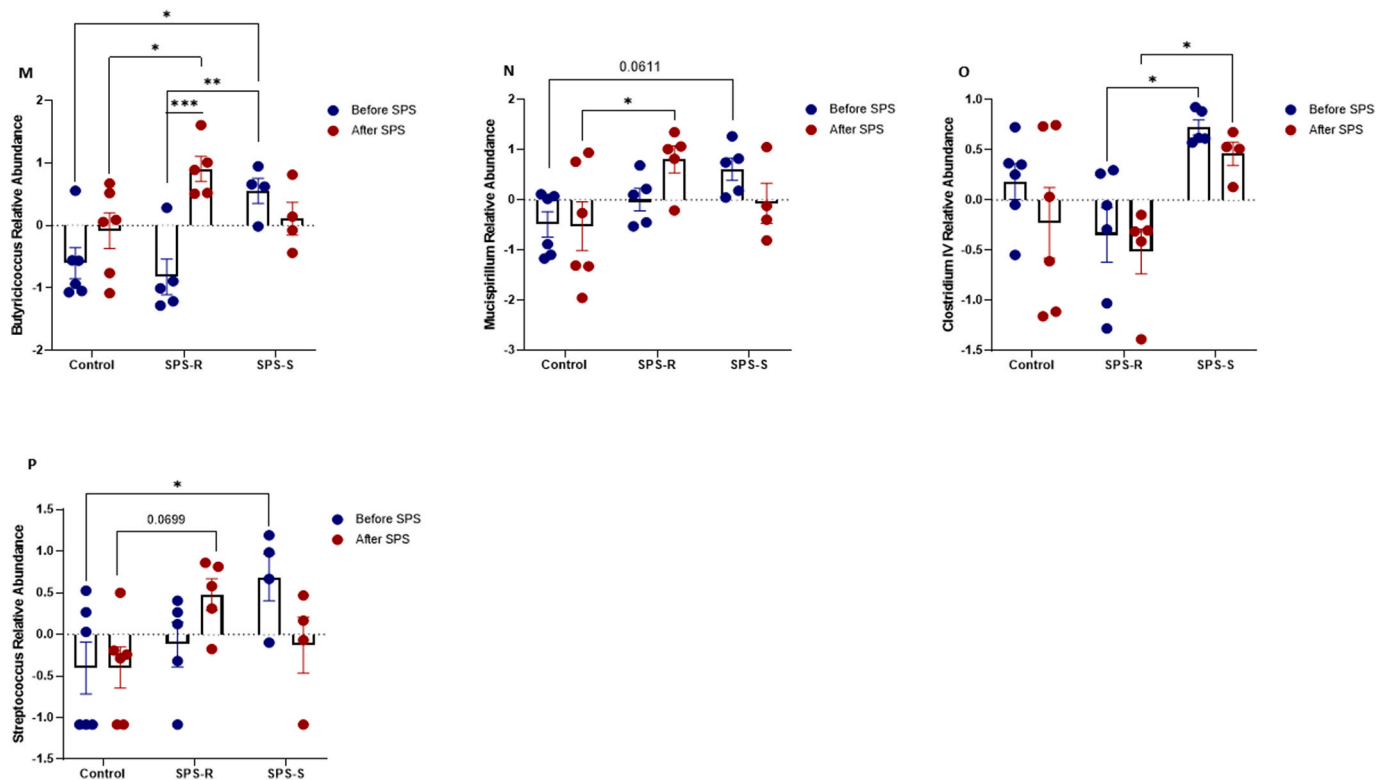
Fig. 6. (*continued*).

urinary epinephrine decreased significantly in both subgroups (SPS-R, p=<0.0001, SPS-S p=<0.0001) and did not differ from the controls (Fig. 8A).

As for norepinephrine, the basal level differences among the groups did not reach significance, yet 90 min into immobilization, the levels increased significantly in SPS-S subgroup (p = 0.032) and trended towards increase in the SPS-R subgroup (p = 0.07). Again, the increase tended to be higher in SPS-S subgroup compared to the SPS-R subgroup (p = 0.082). Finally, on the day of sacrifice, the levels decreased significantly in SPS-S subgroup relative to the previous time points (basal levels (p = 0.0181) and 90 min into immobilization (p < 0.0001)), with no differences observed among the groups (Fig. 8B).

3.4. SPS altered expression of brain tight junction proteins in SPS-S subgroup

Gut microbiota is also regarded as a potential regulator of blood brain barrier (BBB) permeability, through modulating the expression of tight junction (TJ) proteins (Braniste et al., 2014). Since two different behavioral phenotypes were obtained in response to SPS, each with different gut microbial communities, we assessed the expression of two TJ proteins, claudin-5 and occludin, in vHipp. and mPFC. The SPS-S subgroup showed significantly lower expression of claudin-5 in the vHipp ($F_{(2,15)}$ = 3.73, p = 0.048) relative to the SPS-R subgroup (p = 0.0349) (Fig. 9A) and in the mPFC (H (3) = 10.71, p = 0.0005) relative to the controls (p = 0.0043) (Fig. 9B), indicating a higher BBB permeability. Interestingly volatility also correlated with the expression levels of claudin-5 in the ventral hippocampus (r = 0.5, p = 0.0375) (Fig. 9C). No differences were found in the expression of occludin among the groups in either of the brain regions analyzed (Fig. 9D and E).

3.5. SPS-S cohort exhibit shorter colon length

Several studies have shown that a shorter colon is associated with inflammation and colonic inflammation induces depressive and anxiety-

like behaviors (Poritz et al., 2007; Chen et al., 2015a,b). In this experiment, differences in colon length among the groups approached significance ($F_{(2,15)}$ = 3.47, p = 0.058), and Tukey's multiple comparisons test showed a significantly shorter colon in the SPS-S subgroup relative to the controls (p = 0.0478) (Fig. 10).

3.6. Body weight, cecum weight and cecal SCFA quantification

It has been reported that exposure to stress interferes with the host's metabolism and body weight, causing loss of weight or slower weight gain in stressed animals compared to unstressed controls (Harris et al., 2002). However, in our experiments there was no difference in the net weight gain among the groups measured on day 15 and 32 after SPS (Fig. S7A). The cecal weight was also similar among the groups (Fig. S7B).

However, the cecal SCFA levels were different. The levels of acetate ($F_{(2,12)}$ = 7.597, p = 0.0074), one of the major forms of SCFA, were significantly lower in SPS-S subgroup relative to the SPS-R subgroup (p = 0.0062) and trended towards decrease relative to the controls (p = 0.0670) (Fig. 11A). Strong inverse Pearson's correlation was also observed between acetate levels and Anxiety Index on the EPM (r = −0.89, p = 0.0005) (Fig. 11B). No differences in the levels of Butyrate and Propionate were found among the groups (Fig. 11C and D). As for the minor forms of SCFA, one-way ANOVA revealed significant differences in the levels of valerate ($F_{(2, 12)}$ = 4, p = 0.030) and caproate ($F_{(2,12)}$ = 4, p = 0.038). Relative to the controls, the SPS-R subgroup had a trend of decreased levels of valerate (p = 0.0513), whereas the SPS-S subgroup had significantly lower levels of valerate (p = 0.0468) and caproate (p = 0.0413) (Fig. 11E and F).

4. Discussion

The current study provides a proof of concept that inter-individual differences in the microbial signatures may predispose the host to resilience (animals harboring microbiota with an overall anti-
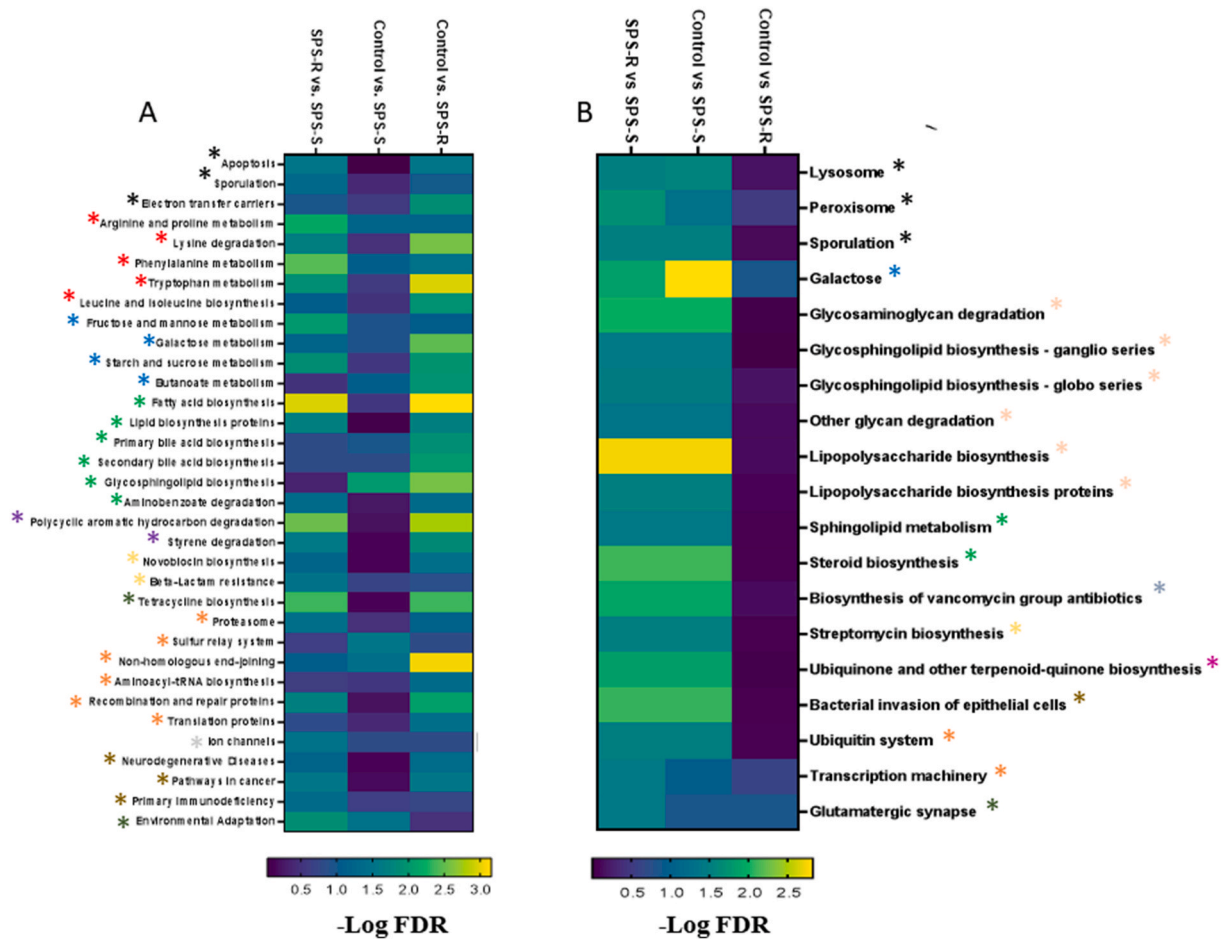
Fig. 7. *Predictive functionality of gut microbiota before and after SPS* Heat map showing the significantly different KEGG pathways among the cohorts **(A)** Before SPS. **(B)** After SPS. Lipid metabolism, non-homologous end-joining, primary immunodeficiency, novobiocin biosynthesis, galactose metabolism, sphingolipids, galactose, LPS biosynthesis, bacterial invasion into epithelial cells, ubiquinone and other terpenoid-quinone biosynthesis were non-parametrically distributed and were analyzed using Kruskal-Wallis test. The remaining data passed the normality test and were analyzed by one-way ANOVA. Both testes were followed by multiple comparison tests. The comparison between the groups is presented as (-log FDR). Values, 2 SD away from the mean were excluded from the analysis. *Cellular processes, *amino acid metabolism, *carbohydrate metabolism, *lipid metabolism, *xenobiotics metabolism, *glycan metabolism, *biosynthesis of secondary metabolites, *genetic information processing, *environmental information processing, *human diseases, *organismal systems, *co-factors and vitamin metabolites, *terpenoids and polyketides metabolism.
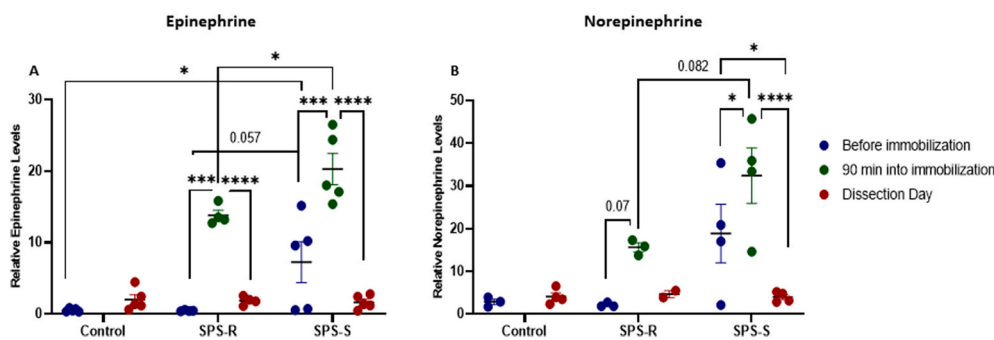

Fig. 8. *Urinary epinephrine and norepinephrine levels before SPS and* 90 min *into immobilization were higher in SPS-S subgroup.* Urine samples were collected before SPS, 90 min into immobilization, and on the day of dissection to measure urinary epinephrine and norepinephrine levels of individual animals. **(A)** Relative Epinephrine levels **(B)** Relative Norepinephrine levels. Data were analyzed by two-way ANOVA repeated measures with post-hoc Šídák's multiple comparisons. Each dot represents value for an individual animal. Blue-Controls, green-SPS-R, red-SPS-S. All data are expressed as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inflammatory phenotype) or susceptibility (animals with pro-inflammatory microbiota) to SPS – triggered behavioral deficits. We further demonstrate that exposure to traumatic stress perturbs the microbiome and shifts the microbial composition in both cohorts. These changes are accompanied with differences in predictive functionality, cecal SCFA levels (index of bacterial metabolic activity), colon length
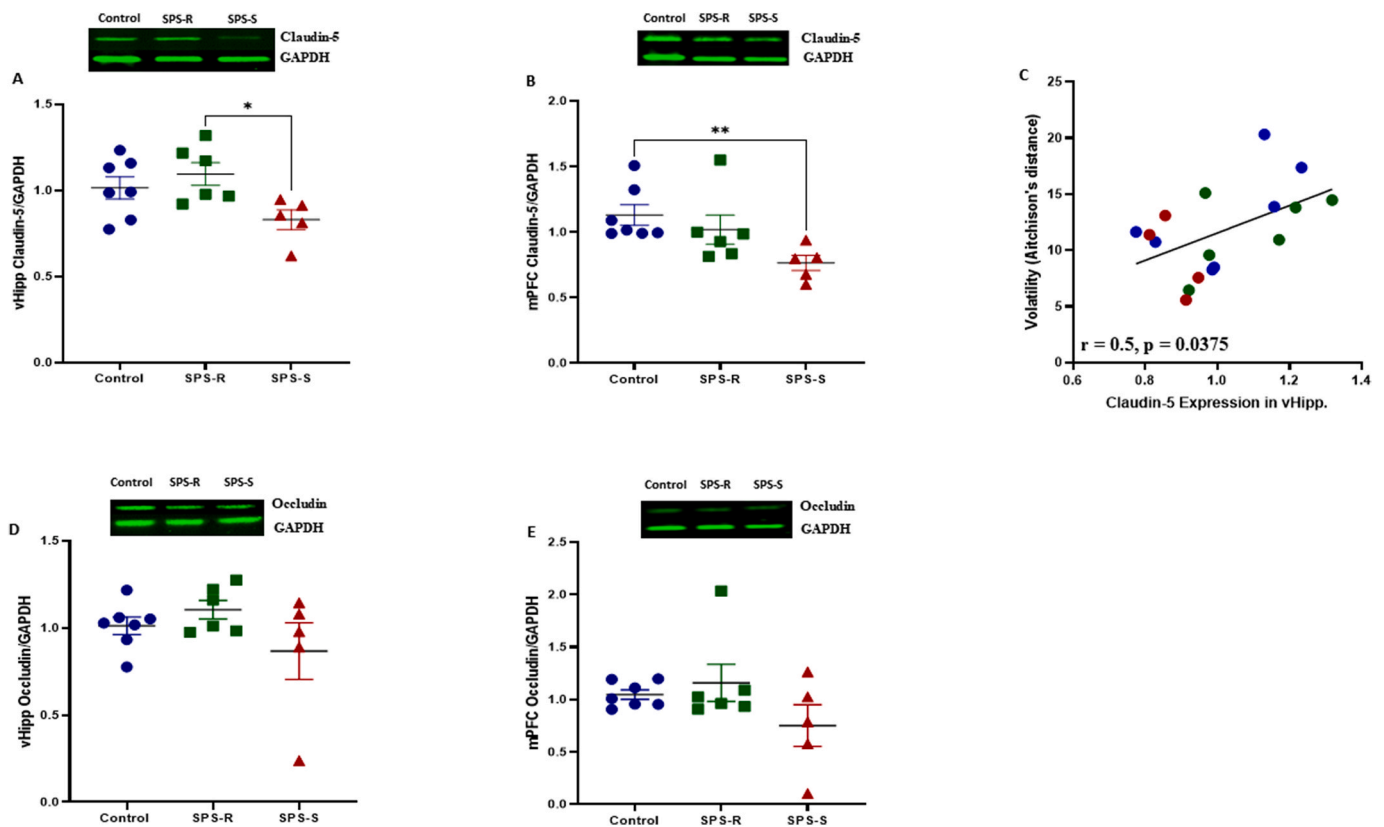
Fig. 9. *Exposure to SPS decreased the expression of brain tight junction protein Claudin-5 in SPS-S subgroup.* Ventral hippocampus (vHipp) and medial prefrontal cortex (mPFC) of each animal were dissected and Western blot was performed to analyze the expression of tight junction proteins. Expression of Claudin-5 in (**A**) ventral hippocampus and (**B**) medial prefrontal cortex, (**C**) correlation between volatility and claudin-5 expression in vHipp; Expression of Occludin in (**D**) ventral hippocampus and (**E**) medial prefrontal cortex. Representative Western blots are shown. Claudin-5 and Occludin protein expression data in mPFC were non-parametrically distributed and were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparison test. Claudin-5 and Occludin protein expression data in hippocampus passed the normality test and were analyzed using one way-ANOVA, followed by Tukey's multiple comparisons test. Each dot represents values for an individual animal. Blue-Controls, green-SPS-R, red-SPS-S. All data are expressed as means ± SEM. ns = not significant, *$p < 0.05$, **$p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
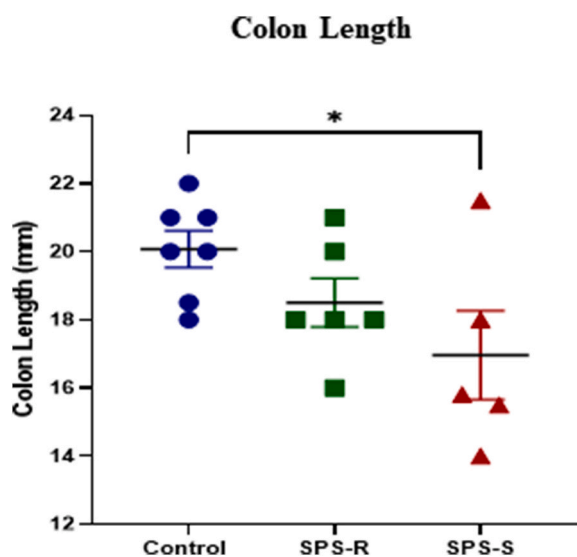

Fig. 10. *Colon length was altered in SPS-S subgroup.* Colonic measurements are expressed as total colon length. The data passed the normality test and were analyzed using one way-ANOVA, followed by Tukey's multiple comparisons test. Each dot represents value for an individual animal. Blue-Controls, Green-SPS-R, Red-SPS-S. All data are expressed as means ± SEM. *$p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(marker of intestinal inflammation), basal and stress-stimulated urinary catecholamine levels (autonomic nervous system input in the gut brain axis), and brain tight junction claudin-5 protein expression levels (index of altered BBB permeability), indicating multi-directional interactions along the gut-brain axis in the individual responses to the traumatic experience.

The results point to pre-existing differences in the gut microbial composition and functional output of outbred rats that relates to their ability to cope with traumatic stress experience. Comparison analysis of the 16S rDNA sequencing data demonstrated separation between SPS-subgroups on a PCA plot and decreased alpha diversity in SPS-R cohort relative to the SPS-S subgroup and the controls. Differences at various taxonomic levels were also observed between SPS-R and SPS-S subgroups, with several genera correlating significantly with the animals' anxiety-like behavior. For example, the relative abundance of Lactobacillus, a well-studied probiotic with health benefits ranging from modulation of the immune system and alleviation of metabolic disorders to regulation of the gut environment (Galdeano and Perdigón, 2004, Lee et al., 2016; Amdekar et al., 2012, Li et al., 2016), was significantly higher in the SPS-R subgroup and correlated inversely with Anxiety Index. Similarly, the abundances of Barnesiella and Bacteroides, both significantly higher in the SPS-R cohort compared to the SPS-S subgroup, also correlated inversely with Anxiety Index. Barnesiella is reported to play a beneficial role in the gut (Ubeda et al., 2013), and its reduced abundance is reported in patients with Crohn's disease, colitis, colorectal cancer, IBD (Mancabelli et al., 2017) and with MDD (Liu et al., 2020). On the other hand, the relative abundance of genera
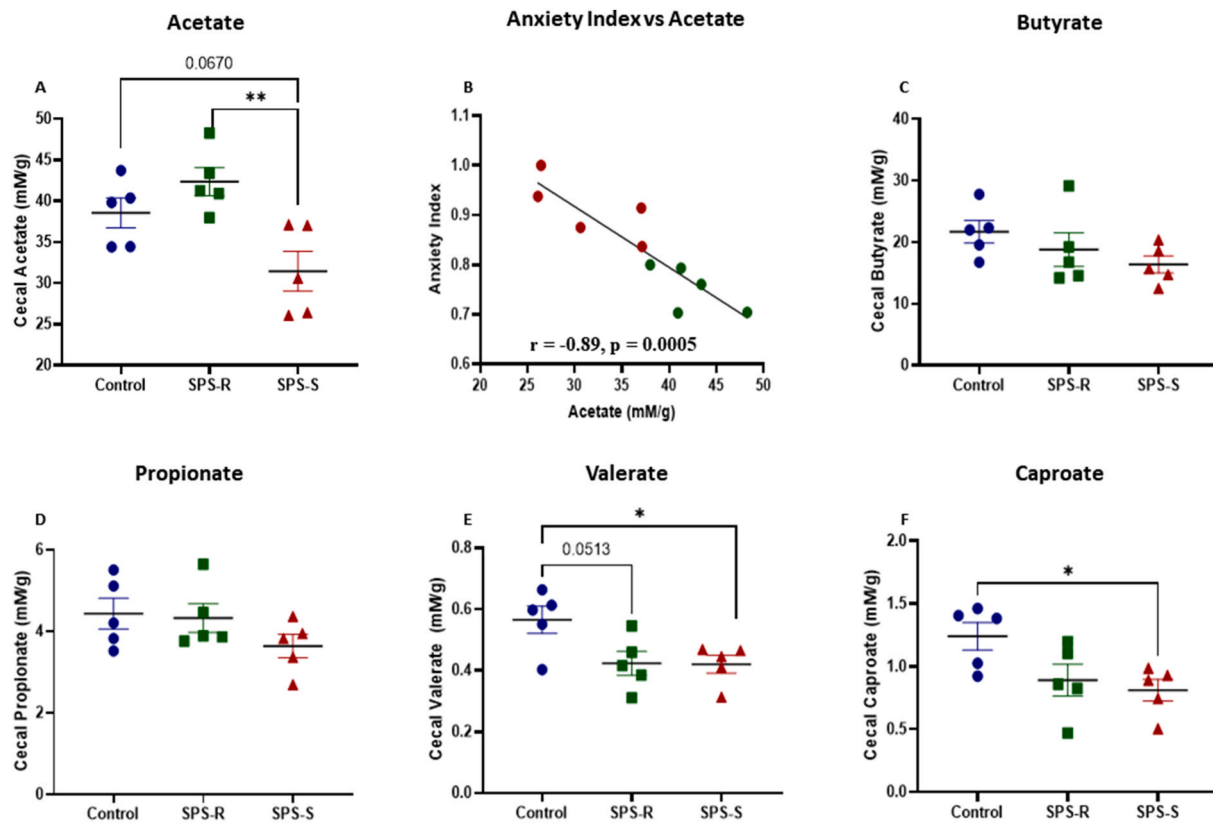
Fig. 11. *SPS altered the levels of cecal SCFA* Cecal content was used for SCFA analysis. (**A**) Levels of Acetate, (**B**) correlation of Anxiety Index with Acetate levels, (**C**) Levels of Butyrate, (**D**) Levels of Propionate, (**E**) Levels of Valerate, (**F**) Levels of Caproate. Data from SCFA levels passed the normality test and were analyzed using one way-ANOVA, followed by Tukey's multiple comparisons test. Blue-Controls, green-SPS-R, red-SPS-S All data are expressed as means ± SEM. *p < 0.05, **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Lachnospiracea-Incertae-Sedis, with known pro-inflammatory characteristics (Jeffery et al., 2012; Jiang et al., 2015; Labus et al., 2017), was higher in the SPS-S subgroup and correlated positively with Anxiety Index. Correlation between Lachnospiracea-Incertae-Sedis' abundance and MDD severity in IBS patients (Li et al., 2018, Schoepfer et al., 2008; Duck et al., 2007) and in patients with autism (Zou et al., 2020, Rea et al., 2020) were also reported. In addition to the dissimilarities observed in the microbial taxonomic composition, several differences in their predictive functionality were also seen before SPS. For instance, SPS-R subgroup's functionality was different from both the SPS-S subgroup and the unstressed controls, with the SPS-R cohort having higher carbohydrate metabolism and genetic information processing, among other pathways. In general, although the characterization of microbiota as harmful or beneficial is still in its initial stages, and contradicting results are reported, the current findings are consistent with our hypothesis that inter-individual differences in the gut microbial composition and function may play a role in predisposing rats to SPS-resilience or vulnerability. However, further studies using fecal microbiota transplantation are needed to prove causality.

Previously, changes in gut microbial communities and in their metabolites after exposure to acute or chronic stress have been reported (rev. in Cryan et al., 2019; Bear et al., 2021; Pearson-Leary et al., 2020), however, the effect of SPS has not been extensively examined. In this study, differences in alpha or beta diversities 15 days after SPS were not seen, contrary to a recent study (Zhou et al., 2020) which reported shifts in these indexes in the SPS group at an earlier time point (7 days after exposure to stress). We chose the two-week time point because behavioral deficits caused by SPS are better demonstrated after 15 days compared to 7 days (Serova et al., 2019b), yet this timeframe may not be optimal for stress-induced compositional changes in the microbiome. Multiple collections of fecal samples at different time points throughout

the experiment will be implemented in future studies to reveal the dynamics in the microbial ecosystem in each cohort after SPS. In this context, it should also be mentioned that, based on evidence from animal models (rodents), the observed compositional changes in the gut microbiota vary widely between studies due to differences in the type of stress, time of analysis, kinetics of recovery following stress and gut niches examined (Bear et al., 2021). In this regard, increased volatility in a chronic social defeat stress model was negatively correlated with social behavior and was suggested to be a marker of stress susceptibility (Bastiaanssen et al., 2021). By the same token, we did not find differences in volatility among the groups, which again might be due to the timeframe of analysis and/or the relatively modest number of animals tested. Yet, volatility correlated inversely with Anxiety Index and duration spent in closed arms, and positively with the time spent on open arms and claudin-5 expression in ventral hippocampus (measures of stress resilience). Along with the observed changes in the gut microbial communities, analysis of predictive functionality of gut microbiota after SPS revealed enhanced bacterial invasion of epithelial cells and LPS/LPS protein biosynthesis pathways in SPS-S subgroup. Thus, the proinflammatory pathways and the shorter colon (indirect marker of inflammation) observed in SPS-S subgroup can contribute to their anxiety-like behavior. Interestingly, however, the lipid and glycan metabolisms increased in SPS-S subgroup and decreased in SPS-R cohort, similar to the shifts observed in the gut microbial communities after SPS.

One of the key mediators of host-microbiota interactions are the SCFA. SCFA are produced mainly in the proximal colon by gut microbiota (Verbeke et al., 2015). In the current study, differences in some of the major and minor forms of SCFA were found among the groups. For instance, the levels of acetate, one of the major forms of SCFA, were significantly altered between the cohorts, with the SPS-S subgroup having significantly lower levels compared to the SPS-R subgroup.

Importantly, there was a strong inverse correlation between cecal acetate and anxiety levels in the SPS-subgroups. Acetate accounts for 60%–70% of the total SCFA and has strong anti-inflammatory properties when used alone or in combination with other SCFA both in animal (Wenzel et al., 2020; Masui et al., 2013) and human studies (Sun et al., 2021; Olsson et al., 2021). It also serves as a critical metabolite required to regulate microglial maturation and function (Erny et al., 2021). Additionally, acetate plays a key role in epigenetics. It is converted into acetyl CoA which then participates in histone acetylation, especially in a state of glucose deprivation or hypoxia. Histone acetylation can have long lasting effects that contribute to a state of depression and anxiety (Dalton et al., 2014). In this context, acetate supplementation by oral gavage, is reported to enhance density of CA1 pyramidal neurons, lower the transcription levels of HDACs, increase the transcription of HAT and boost acetyl-CoA content in the nucleus, and significantly improve depressive-like behavior in the chronic social defeat stress model (Huang et al., 2021). In contrast to acetate, which mainly differed between the SPS-subgroups, the levels of the two less abundant SCFA, valerate and caproate, were lower in both SPS subgroups compared to the controls, however, the decrease was only significant in the SPS-S cohort. Valerate is shown to have anti-inflammatory activity *in vitro* (Tayyeb et al., 2020), and given alone it can reduce the phagocytic activity of microglia-like cells, inhibit cytokine production and histone deacetylase activity (Wenzel et al., 2020).

A key mechanism whereby the gut microbiome impacts brain function is the regulation of BBB permeability (Pfau et al., 2018). The BBB maintains a stable brain microenvironment needed for proper neuronal functioning by serving as a functional and structural roadblock to microorganisms, immune cells, and to fluctuations in plasma composition (Abbott et al., 2010, Haddad-Tóvolli et al., 2017). In the current study, we observed decreased expression of claudin-5 in the vHipp and mPFC of the SPS-S subgroup relative to SPS-R or unstressed controls, indicating increased BBB permeability. To our knowledge this is the first study to report altered BBB permeability in the SPS animal model of PTSD. Reduced expression of claudin-5 protein has been reported in depressed patients (Greene et al., 2020) and in chronic social stress-susceptible mice (Menard et al., 2017). Moreover, the downregulation of claudin-5 alone was shown to be sufficient to induce depressive-like behavior (Menard et al., 2017), and administration of butyrate or propionate and acetate, or the bacteria which produces them was enough to restore its expression. Yet, neither butyrate nor the butyrate producing bacteria had any effect on claudin-5 expression (Braniste et al., 2014). Collectively these studies are consistent with our findings, which demonstrate lower levels of acetate and claudin-5 protein, despite having similar levels of butyrate in the SPS-S cohort. Moreover, the levels of acetate strongly and inversely correlated with the Anxiety Index, further emphasizing the vital role played by acetate in coping with stress-triggered behavioral deficits.

Finally, we observed higher urinary epinephrine and norepinephrine levels in the SPS-S cohort compared to the controls and SPS-R subgroup prior to SPS as well as 90 min into the immobilization. One of the most consistent findings in PTSD is an increased catecholaminergic activity both centrally and peripherally. For instance, PTSD patients have higher urinary catecholamine excretion than control subjects or subjects with other psychiatric disorders (Kosten et al., 1987; Pitman and Orr, 1990, Yehuda et al., 1992). Moreover, urinary epinephrine collected from children within 12 h of admission to a trauma center was associated with acute PTSD symptoms (Delahanty et al., 2005). Thus, our results support the notion that the urinary catecholamines might be helpful as an early biomarker of susceptibility to determine subsequent consequences of severe stress. In fact, sympathetic activation as measured by skin conductance in the immediate aftermath of trauma appears to be an early predictor of future PTSD symptoms (Hinrichs et al., 2019). Catecholamines can regulate the GI tract. They can suppress the immune system, stimulate bacterial growth, enhance expression of genes required for virulence, increase the intestinal mucosa adherence to

mammalian gut tissues and invasiveness of pathogens, and alter the secretion of gut microbial products (Mittal et al., 2017, Sandrini et al., 2015). Similarly, accumulating evidence is highlighting the ability of microbes residing in the gut to influence the function of sympatho-adrenal medullary system (Giri et al., 2019; LaGamma et al., 2021). For instance, Nod1 ligands, released from commensal bacteria in the gut were shown to optimize catecholamines secretion, especially epinephrine, from adrenal chromaffin cells during immobilization stress (Xiang et al., 2021). Similarly, alterations in gut microbial composition of Brandt's volve due to cold temperature stress influenced the release of norepinephrine in the intestine and the brown adipose tissues (Bo et al., 2019). Further studies are needed to clarify the complex bi-directional communication between the peripheral nervous system and gut microbiome, and their effect on the subjects' behavioral outcomes after exposure to traumatic experience.

While this study was designed to test a proof of principle, there are some limitations worthwhile discussing: we used 16S rDNA sequencing which produces reliable taxonomic classifications and relative abundances but has limited taxonomic and functional resolution as compared to high cost, shotgun metagenome sequencing. In addition, fecal samples for microbiome profiling were collected before and after SPS exposure, but a week earlier than the time of sacrifice and tissue dissection. The experiment was designed as such to capture more immediate post stress changes in the microbiome (and exclude as much as possible the potential effects of the behavioral tests). In addition, bacterial metabolites (SCFA), BBB permeability and colon length were analyzed at one time point (after sacrifice). Thus, we cannot determine whether these differences between the cohorts pre-existed or resulted from exposure to SPS. Lastly, like in most studies examining the effect of stress on the bidirectional gut-brain communications, our experiments were performed with male rats. However, females are twice as likely to develop anxiety disorders and PTSD following a traumatic stress (Green et al., 2019), and the microbiota is an important environmental factor that may account for differences between men and women in neurologic diseases (Jaggar et al., 2020). Thus, the study should be expanded to include female rats. Given the observational study design, our data enabled the identification of associative (and not necessarily causative) effects of variations in microbiome composition, function, and metabolic activity and behavioral outcomes in stress-resilient and susceptible animals. Future mechanistic experimental validation is required to substantiate these findings.

In conclusion, the findings reveal that there are differences in the microbiota, assessed in fecal samples, and in urinary epinephrine/norepinephrine levels prior to exposure to SPS. If translatable to humans, this may provide non-invasive biomarkers or assays to assess the potential risk of developing traumatic stress triggered neuropsychiatric disorders. This could also help to reduce the number of subjects needed in clinical studies for prevention of PTSD by confining the treatment to only the susceptible individuals.

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Author Contribution

Arax Tanelian: planning, experimentation, analysis, writing the manuscript: Bistra Nankova: planning, experimentation, analysis, writing the manuscript; Mariam Miari: Bioinformatics analysis; Roxanna Nahvi: experimentation, review of manuscript; Esther Sabban: conceptualization, supervision of experimentation, analysis and writing. All authors read and approved the submitted manuscript.

Declaration of competing interest

The authors declare that the research was conducted in the absence

of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2022.100461.

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