Fecal microbiota transplantation (FMT) from healthy and bipolar donors elicits distinct emotional behaviors and gut-brain metabolite profiles in mice

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Abstract

Bipolar disorder (BD) is a chronic mood disorder characterized by recurrent episodes of depression and (hypo-) mania. Fecal microbiota transplantation (FMT) is a powerful translational tool for investigating the connection between the gut microbiome and BD, and there is evidence FMT can transfer affective symptoms of BD from humans to mice. In this study, we compared the behavior, gut-brain metabolomic profiles, and inflammatory marker expression in two groups of adult female C57BL/6 mice, one receiving FMT from a human donor with BD in a mixed episode (YMRS = 14, HAM-D = 20) and another receiving FMT from a weight and age matched control donor without BD (HAM-D and YMRS = 0). Here we demonstrate that mice receiving FMT from individuals with BD had an increased abundance of the Bacteroidota phylum and decreased abundances of *Parabacteroides merdae* and *Akkermansia muciniphila* associated with altered levels of fecal metabolites, short-chain fatty acids, and related gut hormone expression relative to controls. BD mice also exhibited differential regulation of several metabolites and inflammatory markers in the amygdala, with glycine being the most prominently affected. Furthermore, BD mice exhibited increased anxiety-like behavior and decreased sociability, indicating that some aspects of the behavioral phenotype of BD are transferable from humans to mice via FMT. Taken together, these findings implicate the involvement of gut-brain signaling in the physiological and behavioral changes observed in our BD-FMT mouse model, and provide a promising translational framework for understanding the mechanisms of BD.

Introduction

Bipolar disorder (BD) is a chronic mood disorder characterized by recurrent episodes of depression, hypomania, and mania. BD is one of the most common psychiatric disorders worldwide, with an estimated lifetime prevalence of 1.06% for BD1 and 1.57% for BD2 [1,2]. BD can be debilitating and even fatal, with suicide being 10-30% more likely for BD patients than the general population [3]. The exact biological mechanisms underlying BD are not completely elucidated. Even though the psychiatric genomic consortium (PGC) revealed 64 genome-wide significant gene variants predisposing for BD [4], the multifactorial phenotype of BD cannot be explained by genetics alone. Biopsychosocial interactions are necessary for the outbreak of the disorder, and gene-environment-interactions are also crucial for its progression.

The gut microbiome is a highly promising avenue through which metabolic signaling, inflammatory pathways, environmental factors, and genetics all influence BD pathogenesis. The gut microbiome has been suggested to be involved in numerous central nervous system (CNS) disorders such as major depressive disorder (MDD) [5,6], anxiety [7–10], schizophrenia [11], and attention-deficit/hyperactivity disorder (ADHD) [12] by way of the bidirectional signaling pathway referred to as the “gut-brain-axis.” Intestinal microbiota play a prominent role in gut-brain signaling by producing metabolites such as short-chain fatty acids (SCFAs) that regulate CNS activity through interception of the vagus nerve [13–15]. As a result, gut microbiota can contribute to the regulation of the neuromodulatory and neuroinflammatory systems implicated in the pathogenesis of various psychiatric disorders [16]. There is considerable evidence that the gut microbiome plays a mechanistic role in BD. BD patients have been found to

harbor different intestinal bacterial populations and have lower gut microbial diversity than healthy controls [17]. Since intestinal microbiota are highly sensitive to a variety of environmental factors [1,18], disturbance of the gut microbiome by external agents such as traumatic events [19,20], viral infection [21], and climate [22–24] may affect BD etiology as well.

Fecal microbiota transplantation (FMT) may be a powerful translational tool for researching BD. One study found that depression-like behavior is transferable from humans to mice via FMT [25], and another showed that mice receiving FMT from individuals with ADHD exhibited neurological changes and anxiety-related behaviors associated with ADHD [12]. Furthermore, gut microbiota transferred from a human with BD to mice was shown to affect the expression of TRANK1 [26], a gene whose down-regulation in the brain is associated with BD neuropathogenesis [27]. FMT also has therapeutic potential. FMT from a healthy human donor alleviated symptoms of alcohol-induced depression and anxiety in mice [28]. In a report involving human patients, FMT from healthy donors was an effective adjunctive treatment for depression for 4-8 weeks [29]. A 2022 case study also found that FMT improved symptoms of depression and mania for a male bipolar patient [30].

Here we used FMT as a translational tool to research the influence of gut-brain signaling on BD pathogenesis in a mouse model. We compared the behavior, gut-brain metabolite profiles, and inflammatory marker expression of two mouse groups, one receiving FMT from a human donor experiencing a mixed bipolar episode and the other receiving FMT from a healthy age and weight-matched donor without BD. Given the potential role of the gut-brain axis in BD pathogenesis, we hypothesized that FMT from

a human donor with BD to mice would elicit distinct behavior, inflammatory marker expression, and gut-brain metabolomics from mice receiving FMT from a control donor.

Materials and methods

Animals

All experiments were approved by the ethical committee at the Federal Ministry of Education, Science and Research of the Republic of Austria (permit BMBWF-66.010/0047-V/3b/2019). Three cohorts of female C57BL/6J mice were bred in house and raised at a controlled temperature (22°C) and illumination (12:12 hr light-dark cycle, lights on at 07:00), and given access to water and a standard chow diet ad libitum.

Donors and fecal sample processing

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Medical University of Graz (protocol code 31-120 ex 18/19, date of approval: 27.03.2019). Informed consent was obtained from all subjects involved in the study. The Young Mania Rating Scale (YMRS) was used to assess the severity of manic symptoms [31], and the Hamilton Depression Scale for Depression (HAM-D) was used to assess the severity of depressive symptoms [32] in human donors. Fecal samples were collected from a female human donor in a mixed episode of bipolar disorder with a YMRS score of 14 and a HAM-D score of 20, as well as from a weight and age matched healthy control with HAM-D and YMRS scores of 0. The donor with BD was only on a very low dose of venlafaxine (37.5 mg/d) and no

mood stabilizers, which resembles an almost drug native state. The patient gave informed consent to our longitudinal study before in euthymic state. Exclusion criteria for both groups included antibiotic or antifungal treatment within the previous month, regular intake of prebiotics or probiotics, acute or chronic somatic diseases or infections (including rheumatoid arthritis, systemic lupus erythematosus, neurodegenerative and neuroinflammatory disorders), severe alcohol or drug abuse, dementia, history of digestive disease such as inflammatory bowel disease and irritable bowel syndrome, history of gastrointestinal surgery (other than appendectomy), current pregnancy or breast-feeding, history of eating disorder (anorexia, bulimia) in the last two years, and laxative abuse. Fecal samples were collected in a plastic container containing an anaerobic generator (AnaeroGenTM 2.5L, Thermo Fisher Sci-entific). 60 to 100 g of the samples were processed within 6 hours of collection in an anaerobic chamber (Whitley A85 Workstation, Don Whitley Scientific). The stool samples were diluted in 150 mL of anaerobic, reduced sterile phosphate-buffered saline (PBS), homogenized and filtered through a sieve to remove solid components from the sample. 10% glycerol was added and the sample was aliquoted into 4 mL anaerobic tubes (Anaerobic Hungate Culture Tubes, Chemglass Life Sciences) [33]. Samples were stored at -70°C until stool transfer.

Fecal microbiota transplantation (FMT)

Three cohorts of 20-30 week old female C57BL/6J mice were treated with antibiotics added to their drinking water (0.5 mg/mL neomycin, 1 mg/mL meropenem, and 0.3 mg/mL vancomycin) for 1 week to deplete their existing gut microbiome and promote

the uptake of donor microbiota as previously described [34]. A day prior to FMT, mice were fasted to prevent fecal blockage in the colon during transplantation and were given normal tap water instead of antibiotics. While anesthetized with isoflurane, one group of mice received stool from the bipolar donor and another group was transferred stool from the healthy control. Approximately 0.2 mL of human stool sample was administered rectally into each mouse. A few drops of stool were also placed in the cage and on a few chow pellets to promote further microbiota uptake.

Open Field Test

Cohort 1 mice were subject to the open field test 6 days post FMT as previously described [34]. The open field apparatus was a 35 lux illuminated 50 × 50 × 40 cm opaque, gray plastic box. Mice were placed in one corner of the open field and their locomotion (distance, velocity, speed) was measured for 5 minutes using a video camera and “EthoVision” (Noldus, the Netherlands) software. The open field area was divided into a 36 × 36 cm center zone and the surrounding periphery zone. Entry into either zone was defined by the center point of the mouse.

Elevated plus maze

Mice in cohort 1 were subject to the elevated plus maze (EPM) 10 days post FMT as previously described [34]. The EPM apparatus comprised two open arms (30 × 5 cm), two closed arms (30 × 5 x 15.5 cm), and a center platform (5 cm × 5 cm). The maze was raised 60 cm above the floor and illuminated 35 lux from above. Time spent in the

open arm, number of entries to open arm, and total distance traveled were recorded over the course of 5 minutes with “EthoVision” (Noldus, the Netherlands) software.

Light/dark box test

Mice in cohort 1 were subject to the light/dark box test (LDT) 14 days post FMT [34]. The light-dark box is a cage (37 × 21 × 20.5 cm) divided into two sections by a partition containing a door (4.5 × 6 cm) (TSE Systems). The light compartment (18.5 × 21 cm) was made of transparent walls and brightly illuminated (350-400 lux), whereas the dark compartment (18.5 × 21 cm) was made of black acrylic walls (20 lux). Mice were placed in the light compartment facing the opening to the dark compartment, and locomotion and exploration of the animals were tracked via two external infrared frames, which recorded light beam interruptions (counts) during a 10-minute test period. Data was analyzed with LabMaster software (TSE Systems, Bad Homburg, Germany).

Social interaction test

Mice in cohort 2 were subject to the social interaction test (SIT) 10 days post FMT. The test was performed in an open field apparatus containing two wire cages divided by a wooden panel extending halfway down the open field box. The SIT consisted of a 5 minute habituation phase and a 5 minute test phase during which the test mouse was exposed to a stranger mouse from a different home cage. The cage placement (right or left) of the stranger mouse was alternated between sessions. The time the test mouse spent in the vicinity (3 cm radius) of the stranger mouse’s cage vs the time it spent in the vicinity of the empty cage was measured with “EthoVision” (Noldus, the

Netherlands) software, using the nose point of the test mouse as a reference. Each test mouse was given a one-hour interval between the habituation phase and the stranger mouse phase.

Splash test

Mice in cohort 2 were subject to the splash test 11 days post FMT to assess depression-like-behavior based on the observation that mice exhibiting depression-like-behavior groom themselves less frequently [34]. Mice were placed in individual cages and sprayed with a 10% sucrose and tap water solution from a spray bottle to promote grooming behavior. Grooming behavior was quantified by latency, duration, and counts of both head grooming and total body grooming. The mice were recorded with a video camera for 5 minutes after being sprayed, and grooming behavior was tracked with “EthoVision” (Noldus, the Netherlands) software.

LabMaster cages

The spanned activity and sucrose preference were recorded continuously from 7 to 14 days post FMT for BD and control mice in cohort 3 using the LabMaster system (TSE Systems, Bad Homburg, Germany) as previously described [35]. The set-up consisted of six 42 x 26.5 x 15 cm test cages with two external infrared frames and a lid attached to two weight transducers. The hardware sampling rate was 100 Hz at the infrared frames and 1 Hz at the drinking and feeding sensors. The sampling interval of the LabMaster software was 1 minute. The two weight transducers were used to measure food and water intake. One drinking bottle was filled with water and another with a 1%

sucrose solution. To record locomotion, the two external infrared frames were placed 4.3 cm apart horizontally, with the lower frame 2.0 cm above the bottom of the cage. The bottom infrared frame recorded horizontal locomotion (ambulatory movements) of the mice, while the top infrared frame recorded vertical movements (rearing, exploration). Mouse activity was measured by counts of interruptions of the light beams of the infrared frames.

Tissue extraction

Tissue extraction was performed as described [36]. Mice were deeply anesthetized with pentobarbital (150 mg/kg) via intraperitoneal injection (IP). Brains were dissected and immediately frozen for 5 seconds in 2-methylbutane on dry ice. The amygdalas were microdissected under a stereomicroscope (Bregma -0.58 to -2.54) on a -20 °C cold plate (Weinkauf Medizintechnik, Forchheim, Germany) [37]. RNase AWAY (Carl Roth, Karlsruhe, Germany) was used to clean dissection instruments before and in between uses. Microdissected brain samples were placed in micro packaging tubes with Precellys beads (Peqlab, Erlangen, Germany) and stored at -70 °C until further analysis. Colon contents and distal and proximal regions were frozen on dry ice and stored at -70°C.

Microbiome analysis

Bacterial DNA was extracted from fecal contents with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. PCR amplification and sequencing was performed by Novogene Europe (Cambridge, UK).

The variable V3–V4 region of the bacterial 16S rRNA gene was amplified with PCR using oligonucleotide primers 341F: 5-CCTAYGGGRBGCASCAG and 806R: 5-GGACTACNNGGGTATCTAAT, while additionally including a molecular barcode sequence. PCR reactions were carried out using the Phusion High-Fidelity PCR Master Mix (New England Biolabs). The PCR products were selected by 2% agarose gel electrophoresis. Same amount of PCR products from each sample was pooled, end-repaired, A-tailed and further ligated with Illumina adapters. Libraries were sequenced on a paired-end Illumina platform (NovaSeq PE250) to generate 250 bp paired-end raw reads. Paired-end reads were assigned to samples based on their unique barcodes and trimmed by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH (V1.2.7) [38]. Quality filtering was performed according to the Qiime quality control process [39] and chimeras were removed using UCHIME algorithm. Sequencing analyses were performed by Uparse software (Uparse v7.0.1090) [40] and sequences with ≥97% similarity were assigned to the same OTUs. Species annotation was performed by QIIME2 software using the annotation database SILVA138. Alpha diversity was calculated for observed species. The QIIME2 tool on the local Galaxy instance (https://galaxy.medunigraz.at/) was used to calculate beta diversity (weighted Unifrac) and predict bacterial biological pathways using PICRUst2.

Metabolomics

Fecal and amygdala samples collected 13 days post FMT from cohort 1 were prepared for NMR spectroscopy measurements as previously described [41]. 50 to 100 mg of

stool or brain tissue were mixed with 200 µl water and 400 µl methanol to inactivate and precipitate proteins. Remaining solids were lysed using a Precellys homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France), stored at -20 °C for 1 hour, followed by centrifugation for 30 min at 10,000 x g at 4 °C. Finally, the supernatants were lyophilized, resuspended in 500 µl NMR buffer (0.08 M Na_2HPO_4, 5 mM 3-(trimethylsilyl) propionic acid-2,2,3,3-d_4 sodium salt (TSP), 0.04 (w/v) % NaN_3 in D_2O, with pH adjusted to 7.4 with HCl or NaOH, respectively), and transferred into 5-mm NMR tubes for measurement on the NMR instrument using the CPMG pulse sequence as described. Spectra pre-processing and data analysis have been carried out using Matlab® scripts (group of Prof. Jeremy Nicholson at Imperials College London). NMR data were imported to Matlab® vR2014b (Mathworks, Natick, Massachusetts, USA), regions around the water, TSP, and remaining methanol signals excluded, aligned, and corrected for sample metabolite dilution by probabilistic quotient normalization [42]. Reported values correspond to arbitrary units (A.U.) derived from the area under the peak being proportional to concentration.

Quantitative polymerase chain reaction

RNA from the colon and amygdala was extracted with the RNeasy Tissue Mini Kit (Qiagen) and RNeasy Lipid Tissue Mini Kit (Qiagen), respectively. Aliquots of 1 µg RNA were reverse-transcribed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). For relative quantification of mRNA, real time PCR was performed with the CFX384 Touch Real-Time PCR System (Biorad) using TaqMan inventoried gene expression assays for Tnf (Mm 00443258_m1), Il1b

(Mm 00434228_m1), Il6 (Mm 00446190_m1), Ifng (Mm 01168134_m1), Ido1 (Mm 00492590_m1), Il10 (Mm 01288386_m1), Ccl2 (Mm 00441242_m1), Chil3 (Mm 00657889_mH), Iba1 (Mm 00479862_g1), Cd68 (Mm 03047343_m1), Arg1 (Mm 00475988_m1), Cldn1 (Mm 00516701_m1), Cldn5 (Mm 00727012_s1), Tjp1 (Mm 00493699_m1), Ocln (Mm 00500912_m1), Glra1 (Mm00445061_m1), Glra2 (Mm01168376_m1), Glra3 (Mm00475507_m1), Glra4 (Mm00501674_m1), Glrb (Mm00439140_m1), Gpr158 (Mm00558878_m1) and the TaqMan Gene Expression Master Mix (Life Technologies). All samples were measured as duplicates. Actb, Gapdh and Ppil3 were used as reference genes. Quantitative measurements of target gene levels relative to controls were performed with the 2-ΔΔCt method using the mean value of the control group as the calibrator [43].

Statistics

Differences among mouse groups were assessed by Student's t-test, two-way ANOVA or repeated-measures ANOVA combined with Sidak post hoc analysis where appropriate. Pearson's correlation was calculated for bacterial taxa that differed between BD and control mice versus behavioral parameters of the EPM followed by Bonferroni correction. Statistical analyses were performed with GraphPad Prism 9 (GraphPad Software, Inc CA, USA). *Metastats* was used for detecting differentially abundant microbial features [44]. Linear discriminant analysis (LDA) Effect Size (LEfSe) calculations implemented in Galaxy were performed to identify the predicted pathways that differentiate FMT effects. The statistical analysis of the metabolomics data was done using the web-based analysis platform “MetaboAnalyst”

(https://www.metaboanalyst.ca/, Version 5.0, last visited 11/3/2023). Statistical significance was defined as P ≤.05.

Results

Compositional and functional microbiome profiles of bipolar and control microbiota recipient mice

Mice receiving bipolar (BD) and control FMT exhibited distinct compositional and functional microbiome profiles 10 days post FMT. Principal coordinate analysis (PCoA) of fecal 16S rRNA expression data demonstrated that the gut microbiomes of BD and control mice clustered differently, as did the microbiomes of their respective human donors (Fig. 1A). Full microbiome engraftment did not occur which is unsurprising given innate differences between human and murine intestinal tracts. 16S rRNA sequencing of fecal samples showed notable distinctions in microbiome compositions between BD and control mice (Fig. 1B). Compared to controls, BD mice contained a significantly higher abundance of the phylum Bacteroidota and a significantly lower abundances of *Akkermansia muciniphila* and *Parabacteroides merdae* (Fig. 1E). Alpha-rarefaction curves approached saturation for both BD and control fecal samples, indicating sufficient sequencing depth to capture microbiome diversity, while no difference in alpha diversity was present between BD and control mice (Fig. 1C). Finally, metagenomic predictions by PICRUSt2 identified differential regulation of several pathways between BD and control mice, elucidating alterations in microbiome functionality between the groups (Fig. 1D).

Assessment of emotional behavior, circadian rhythm, and anhedonia in bipolar and control FMT mice

Mice receiving bipolar FMT exhibited more anxiety-like behavior and decreased sociability than mice with healthy FMT, but no differences in depression-like behavior. During the EPM 10 days post FMT, BD mice spent significantly less time in the open arms (Fig. 2E) and made significantly fewer entries to open arms (Fig. 2F). Time spent in the open arms of EPM also positively correlated with *Akkermansia muciniphila* in both BD and control groups (Fig. 2H). Traveling distance during the EPM was similar between BD and control mice (Fig. 2G). BD mice also displayed less sociability during the social interaction test (SIT) 10 days post FMT. Control mice spent significantly more time in the vicinity of the stranger mouse cage than in the vicinity of the empty cage (Fig. 2J). By contrast, BD mice did not spend a significantly greater amount of time exploring the stranger mouse than the empty cage (Fig. 2J). No significant differences between groups were measured during the OFT 6 days post FMT for time spent in the central area (Fig. 2B), number of entries to the central area (Fig. 2C), or total traveling distance (Fig. 2D). We measured no significant differences in time spent by each group in the light and dark compartments during the light/dark box test (LDT) 11 days post FMT (Fig. 2I). During the splash test 11 days post FMT, no significant difference in grooming duration was recorded between groups (Fig. 2K). No significant differences in circadian activity (Fig. 2L) or sucrose preference at any time point (Fig. 2M) was measured between BD and control mice 7 to 13 days post FMT.

Gut and brain metabolomic profiles of bipolar and control FMT mice

BD and control mice displayed significantly different gut and brain metabolomic profiles 13 days post FMT. Metabolomic analysis revealed significant up-regulation of valeric acid, fructose, glucose, and sucrose in the feces of BD mice relative to controls. Aspartic acid, capric acid, xanthine, and hypoxanthine were significantly down-regulated in BD mice feces (Fig 3A). For short-chain fatty acids, there were significantly lower levels of acetic acid and a notable, but not statistically significant reduction of butyric acid in the feces of BD mice. Levels of fecal propionic and succinic acid were similar between BD and control groups (Fig. 3B). BD mice also had significantly lower levels of fecal L-glutamic acid than control mice (Fig. 3C). Expression levels of gut hormones Peptide YY (*Pyy*) and preproglucagon (*Gcg)* were decreased in the colon of BD mice relative to controls (Fig. 3D). Metabolomic analysis also revealed significant down-regulation of glycine, glycerol, uridine, tryptophan, pyroglutamic acid, inosine, acetic acid, phosphorylcholine, choline, and methionine in the amygdalas of BD mice, with glycine being the most prominently affected. Conversely, adenosine monophosphate and inosinic acid were significantly up-regulated in the BD group (Fig. 3E).

Cytokine, inflammatory marker, tight junction protein, and glycine receptor subunit expression in bipolar and control FMT mice

Thirteen days post FMT, BD and control mice displayed similar levels of colonic (Fig. 4A) and amygdalar (Fig. 4C) *Ido-1* mRNA expression. Pro-inflammatory cytokine (*Tnf*, *Il1b*, *Il6*, *Ccl2*, *Ifng*) and anti-inflammatory cytokine (*Il10*) expression did not differ

significantly between groups in both the colon (Fig. 4A) and amygdala (Fig. 4C). However, BD mice did exhibit a significant increase in amygdalar *Chil3* expression relative to controls (Fig. 4C). There were no significant differences between BD and control mice in the expression of tight junction protein genes *Cldn1*, *Cldn5*, *Tjp1*, and *Ocln* in both the colon (Fig. 4B) and the amygdala (Fig. 4D), nor was there differential expression of microglial markers *Iba1*, *Cd68*, *Cd86*, and *Arg1* (Fig. 4E). *Glra4* expression was significantly increased in the brains of BD mice relative to controls (Fig. 4F). Still, we found no significant differences in the expression of other glycine receptor subunits *Glra1*, *Glra2*, *Glra3*, *Glrb*, and *Gpr158* (Fig. 4F).

Discussion

Our results demonstrate substantial metabolomic, inflammatory, and behavioral distinctions between mice receiving fecal microbiota transplantation (FMT) from a bipolar human donor and mice receiving FMT from a healthy control donor. These include differences in gut microbiome composition, anxiety-like behavior, sociability, fecal and brain metabolite profiles, and expression of inflammatory markers. Taken together, these findings implicate the involvement of gut-brain signaling in the physiological and behavioral changes observed in our BD-FMT mouse model, and provide a promising translational framework for understanding the mechanisms of affective behaviors including BD.

Increased anxiety-like behavior and decreased sociability of BD mice suggest that these behavioral features are especially affected by gut-derived signals. The positive correlation observed between *A. muciniphila* and open arm visits in the

elevated plus maze is supported by previous findings that the relative abundance of *A. muciniphila* negatively correlates with anxiety-related behavior in mice [45]. Furthermore, probiotic treatment with *A. muciniphila* has been shown to reduce anxiety-like behavior, motor degeneration, and learning memory deficits in a mouse model [46–48]. Another murine model of mania demonstrated hyperactivity, disrupted circadian rhythm, and decreased sucrose preference as a measure of anhedonia [49]. However, a lack of significant difference in sucrose preference or circadian activity between BD and control FMT mice suggests that manic and depressive symptoms are less transferable to mice via FMT.

The behavioral differences between BD and control mice are likely linked to differential regulation of several gut and brain metabolites, short-chain fatty acids (SCFAs), and related gut hormone signaling. Reduced *A. muciniphila* in BD mice potentially lowered fecal acetic acid, since *A. muciniphila* produces acetic acid through intestinal mucin degradation [50]. Acetic acid was also decreased in the amygdala, suggesting a link to fecal acetic acid depletion. Elevated fecal valeric acid in BD mice likely resulted from reduced *P. merdae,* which can catabolize branched-chain amino acids such as valine [51]. *P. merdae* is also inversely correlated with depression in humans [52]. Interestingly, increased valeric acid and decreased acetic acid concentrations have been measured in fecal samples from children with autism [53], suggesting a connection between the regulation of these metabolites and neurodevelopmental disorders. Acetate supplementation has also rescued social deficits in an autistic mouse model, implicating decreased central acetic acid levels with impaired social interaction in BD mice [54].

Diminished acetic and butyric acid production in BD mice likely reduced colonic expression of gut hormones *Pyy* and *Gcg* [55,56]. *Pyy* encodes Peptide YY (PYY), a gut hormone produced by endocrine L cells and in pancreatic islets [57] that can affect brain signaling by binding to Y receptors [58]. Notably, *Pyy*-knockout mice exhibit increased depression-like behavior [59]. In mice, the *Gcg* gene encodes for glucagon, but can also produce other hormones such as glucagon-like peptide-1 (GLP-1) [60]. GLP-1 is released from intestinal cells and can circulate in the blood and be absorbed in the brain [61]. GLP-1 attenuated symptoms of mania in a mouse model [62] as well as depressive symptoms in both humans and mice by reducing neuroinflammation [63], ameliorating synaptic dysfunction [64], and promoting neurogenesis [65]. Therefore, altered acetic and butyric acid production in BD mice has significant implications for gut-brain communication and may have influenced mouse behavior through changes in *Pyy* and *Gcg* gene expression.

Our results also indicate metabolic differences between BD and control mice. In BD mice we measured elevated fecal glucose levels associated with predicted decreased glucose-1-phosphate degradation by gut microbiota. Impaired glucose metabolism has been observed in over half of bipolar patients [66], and type II diabetes mellitus and BD are considered comorbid disorders [67]. Elevated levels of sucrose and D-fructose in BD mice further indicate disruptions in gut carbohydrate metabolism that could influence cognitive function [68] and signify neurodegeneration [69]. Reduced fecal concentrations of xanthine and hypoxanthine in BD mice, possibly through enhanced predicted inosine 5'-phosphate degradation by gut microbiota, indicate disrupted purine metabolism, which is likely linked to decreased inosine and

upregulated adenosine monophosphate (AMP) and inosinic acid (IMP) in the amgydalas of BD mice. Interestingly, alterations in purine and adenosine metabolism in the brain are associated with depression [70] and neurodegeneration [69].

An increased abundance of glutamate fermenting *Bacteroides* strains in BD mice could be linked to reduced fecal L-glutamic acid [71], which potentially diminished predicted tetrapyrrole biosynthesis in the gut as well as lowered levels of the glutamic acid derivative, pyroglutamic acid, in the amygdala. Other studies confirm that gut microbiota can influence pyroglutamic acid levels in the brain [72]. Alterations in amygdalar pyroglutamic acid concentrations in BD mice signify disrupted glutamate metabolism and neurotransmission, which is associated with depression and anxiety in both humans and mice [73,74]. In a piglet model, L-glutamic acid supplementation was also shown to improve small intestinal architecture and influence the expression of amino acid receptors and transporters [75], further underscoring the role of reduced fecal L-glutamic acid in gut-brain signaling in our mouse model.

Down-regulation of amygdalar glycine, uridine, choline, glycerol, and L-tryptophan further signify neurodegeneration, cellular membrane breakdown, and abnormalities in synaptic plasticity in BD mice. Amygdalar glycine reductions are potentially linked to diminished fecal sarcosine, since sarcosine inhibits glycine uptake by glycine-transporter 1 (GlyT1) on neighboring glial cells and can cross the blood brain barrier [76]. Inhibition of GlyT1 by sarcosine has even been proposed as a treatment for both MDD [77] and schizophrenia [78]. Alterations in sarcosine and glycine production in BD mice could be linked to predicted up-regulation of the S-adenosyl-L-methionine salvage I pathway in the gut. An increase in amygdalar glycine receptor alpha 4 (*Glra4*)

expression in BD mice is a potential compensatory mechanism to maximize sensitivity to available glycine in the brain. Given that glycine acts as a co-agonist at N-methyl-D-aspartate (NMDA) receptors, glycine level decline could result in abnormalities in synaptic plasticity associated with BD [79]. Of note, glycine supplementation can ameliorate anxiety in rats [80], but elevated glycine in the brain is associated with manic episodes [81], emphasizing the importance of glycine homeostasis.

Because uridine, choline, and glycerol are involved in phospholipid biosynthesis, their down-regulation in the brains of BD mice relative to controls indicates disrupted metabolism of cellular membranes. Importantly, cell membrane dysfunction is linked to the cognitive impairment, reduced neuroplasticity, and neurodegeneration associated with BD [82]. We hypothesize that uridine and glycerol depletion were linked to Bacteroidota increases since some members of the phylum can metabolize glycerol [83] and are positively correlated with uridine supplementation [84]. Predicted up-regulation of pyrimidine deoxyribonucleosides salvage in the microbiomes of BD mice further indicates enhanced uridine metabolism. Our results also suggest that reduced brain choline levels in BD mice are independent of choline regulation in the gut since choline was not significantly increased in the feces of BD mice relative to controls. Interestingly, reinstitution of uridine and choline could mitigate symptoms of BD and related disorders. One study found that uridine supplementation over 7 days significantly increases brain phosphomonoester and phosphodiester levels [85]. Uridine also had dose-dependent antidepressant effects in mice [86] and choline supplementation reduced anxiety symptoms in an autistic mouse model [87].

Amygdalar L-tryptophan down-regulation in BD mice is noteworthy given that tryptophan is an established player in gut-brain signaling. Both BD and MDD are associated with central and peripheral tryptophan depletion [88,89]. In mammals, tryptophan is catabolized into kynurenine (kyn) through the activation of indoleamine 2,3-dioxygenase (IDO), encoded by *Ido-1*. Since *Ido-1* expression was similar in the colons and amygdalas of BD and control mice, amygdalar L-tryptophan depletion was unlikely due to enhanced tryptophan catabolization. Since Bacteroidota are especially enriched in tryptophan metabolizing strains [90,91], increased Bacteroidota in the guts of BD mice could limit L-tryptophan availability. Additionally, fructose malabsorption is linked to lower serum tryptophan concentrations [92], suggesting elevated gut fructose levels as a possible mechanism of tryptophan depletion in BD mice. However, given that fecal L-tryptophan levels were similar between BD and control mice, future work should evaluate possible disruptions in L-tryptophan brain transportation in BD mice.

Furthermore, enhanced amygdalar *Chil3* (chitinase-3-like protein) expression in BD mice is indicative of neuroinflammation. Chil3 is a chitinase-like protein produced by myeloid cells in rodents that is upregulated in the brain in response to neuroinflammation [93–95]. Several studies have linked neuroinflammation to the onset of both depression [96] and bipolar disorder [97]. In humans, Chil3L1 (chitinase-3-like protein 1) is expressed in several cell types including glial cells, and is involved in the inflammatory processes of the innate immune system [98]. Importantly, elevated serum levels of Chil3L1 have been identified in BD patients [99] as well as in individuals with neuroinflammatory conditions [100].

Overall, we demonstrate significant physiological and behavioral differences between mice receiving FMT from a bipolar human donor and those receiving FMT from a healthy, age and weight matched donor. The gut microbiomes of mice receiving BD FMT had an increased relative abundance of Bacteroidota and decreased relative abundances of *P. merdae* and *A. muciniphila*. These differences in microbiome compositions are likely responsible for differential fecal short-chain fatty acid (SCFA) and secondary metabolite production that indicate disrupted carbohydrate metabolism and purinergic signaling in the guts of BD mice. Amygdalar down-regulation of glycine, uridine, choline, glycerol, and L-tryptophan in BD mice further signify altered neurotransmission, neurodegeneration, reduced neural plasticity, and cellular membrane breakdown. Finally, increased *Chil3* expression in the amygdalas of the BD mouse group indicates distinct neuroinflammatory responses, while no significant differences in the colonic and amygdalar expression of cytokines, tight junction proteins, or glial markers were measured between BD and control groups. BD mice also displayed increased anxiety-related behavior during the EPM as well as decreased sociability during the SIT, suggesting that some aspects of the BD behavioral phenotype such as anxiety and a lack of sociability are especially affected by gut-derived signals. Taken together, these suggest a prominent role of gut-brain signaling in bipolar pathogenesis in our mouse model. Future research should delve deeper into the mechanisms of these gut-brain interactions as well perform replication with different FMT donors. Further exploration of the potential therapeutic applications of modifying gut microbiota, either through FMT or probiotic interventions, may reveal novel avenues for BD treatment and offer a more comprehensive understanding of its etiology.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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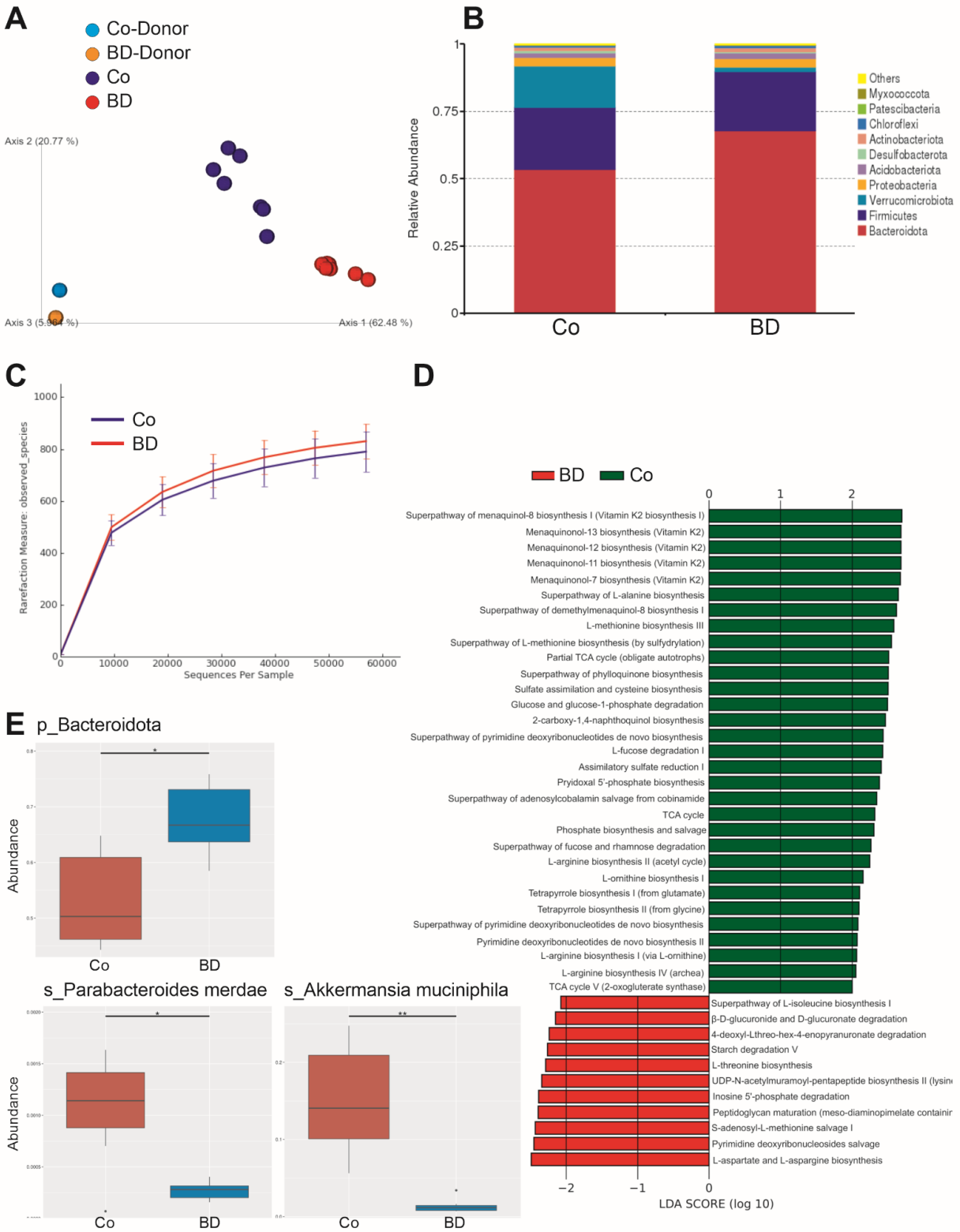

A
Co-Donor
BD-Donor
Co
BD
Axis 2 (20.77 %)
Axis 1 (62.48 %)
B
Relative Abundance
Co
BD
Others
Myxococcota
Patescibacteria
Chloroflexi
Actinobacteriota
Desulfobacterota
Acidobacteriota
Proteobacteria
Verrucomicrobiota
Firmicutes
Bacteroidota
C
Rarefaction Measure: observed_species
Sequences Per Sample
Co
BD
D
BD
Co
Superpathway of menaquinol-8 biosynthesis I (Vitamin K2 biosynthesis I)
Menaquinonol-13 biosynthesis (Vitamin K2)
Menaquinonol-12 biosynthesis (Vitamin K2)
Menaquinonol-11 biosynthesis (Vitamin K2)
Menaquinonol-7 biosynthesis (Vitamin K2)
Superpathway of L-alanine biosynthesis
Superpathway of demethylmenaquinol-8 biosynthesis I
L-methionine biosynthesis III
Superpathway of L-methionine biosynthesis (by sulfydrylation)
Partial TCA cycle (obligate autotrophs)
Superpathway of phylloquinone biosynthesis
Sulfate assimilation and cysteine biosynthesis
Glucose and glucose-1-phosphate degradation
2-carboxy-1,4-naphthoquinol biosynthesis
Superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis
L-fucose degradation I
Assimilatory sulfate reduction I
Pryidoxal 5'-phosphate biosynthesis
Superpathway of adenosylcobalamin salvage from cobinamide
TCA cycle
Phosphate biosynthesis and salvage
Superpathway of fucose and rhamnose degradation
L-arginine biosynthesis II (acetyl cycle)
L-ornithine biosynthesis I
Tetrapyrrole biosynthesis I (from glutamate)
Tetrapyrrole biosynthesis II (from glycine)
Superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis
Pyrimidine deoxyribonucleotides de novo biosynthesis II
L-arginine biosynthesis I (via L-ornithine)
L-arginine biosynthesis IV (archea)
TCA cycle V (2-oxogluterate synthase)
Superpathway of L-isoleucine biosynthesis I
β-D-glucuronide and D-glucuronate degradation
4-deoxyl-Lthreo-hex-4-enopyranuronate degradation
Starch degradation V
L-threonine biosynthesis
UDP-N-acetylmuramoyl-pentapeptide biosynthesis II (lysin
Inosine 5'-phosphate degradation
Peptidoglycan maturation (meso-diaminopimelate containir
S-adenosyl-L-methionine salvage I
Pyrimidine deoxyribonucleosides salvage
L-aspartate and L-aspargine biosynthesis
LDA SCORE (log 10)
E
p_Bacteroidota
s_Parabacteroides merdae
s_Akkermansia muciniphila
Abundance
Co
BD

Fig 1. Compositional and functional microbiome profiles of female C57BL/6J mice 10 days after receiving either bipolar (BD) or control (Co) FMT. PCoA plot of 16S rRNA expression data from feces of control donor (blue), bipolar donor (orange), control mice (purple), and BD mice (red) (**A**). Bar plot showing 16S rRNA relative abundance expression of bacterial phyla in the feces of control and BD mouse groups (**B**). Alpha-rarefaction curves (observed species) for both BD and control fecal samples show no differences in alpha diversity between BD and control mice (**C**). Enzyme Commission (EC) number pathways predicted by PICRUSt2 to be up-regulated in the microbiomes of BD (red) and control (green) mice selected by Linear discriminant analysis Effect Size (LEfSe) (**D**). Bar plot of fecal 16S rRNA sequencing results showing greater abundance of Bacteroidota phylum (q = 0.047) and lower abundance of *Parabacteroides merdae* (q = 0.034) and *Akkermansia muciniphila* species (q = 0.009) in BD mice relative to controls. Significant differences were analyzed using *Metastats* (**E**). BD n = 7, Co n = 7.

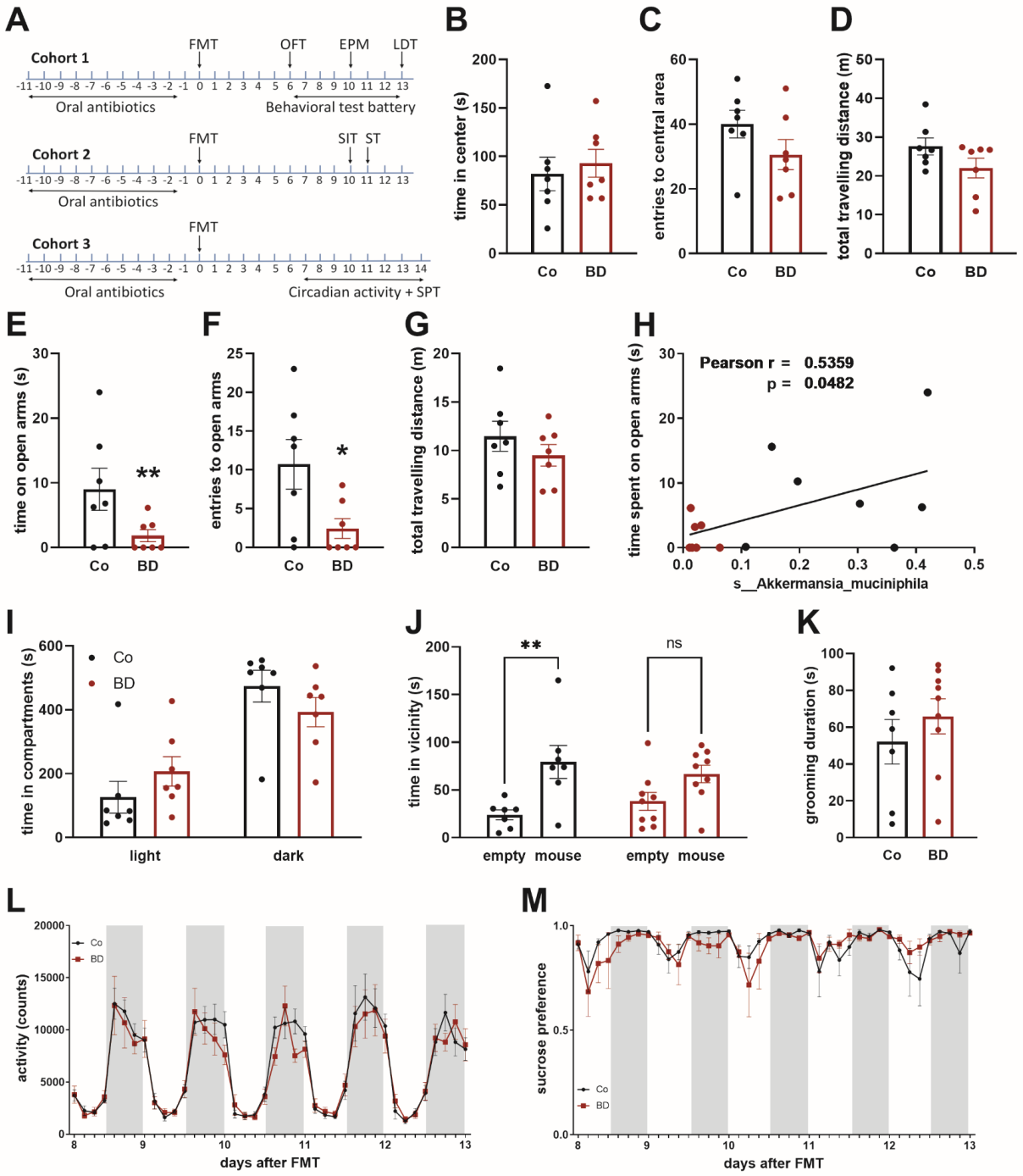
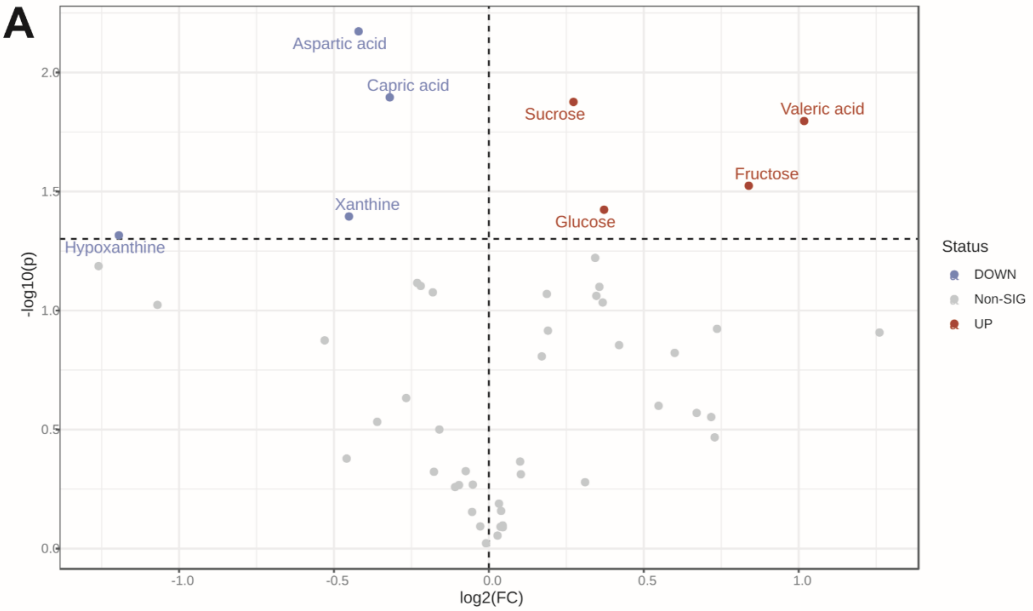
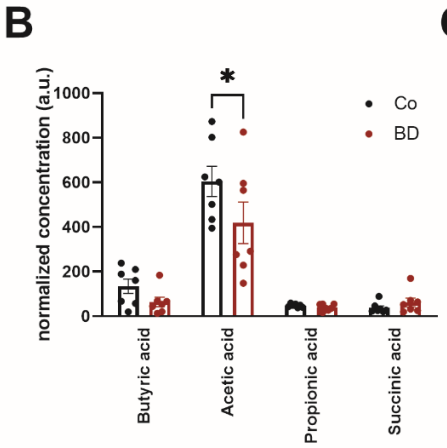
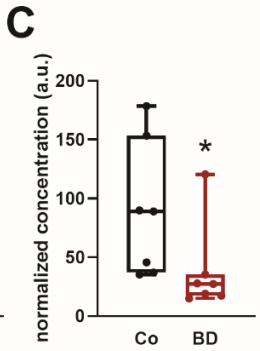


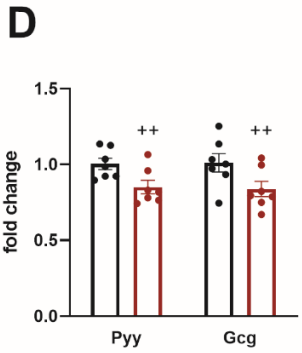
Fig 2. Assessment of emotional behavior, circadian rhythm, and anhedonia in 3 cohorts of mice receiving bipolar (BD) or healthy control (Co) FMT. Experimental timeline, OFT = open field test, EPM = elevated plus maze, LDT = light/dark box test, SIT = social

interaction test, ST = splash test, SPT = sucrose preference test (**A**). Comparison of time spent by BD and control mice in the center of open field (**B**), number of entries to the center of open field (**C**), and total traveling distance during OFT (**D**). Comparison of time spent by BD and control mice in the open arms of EPM ($p = 0.0079$) (**E**), number of entries to open arms ($p = 0.043$) (**F**), and total traveling distance during EPM (**G**). Time spent in the open arms of EPM positively correlates with *Akkermansia muciniphila* relative abundance for both BD and control mice (Pearson $r = 0.5359$, $p = 0.0482$) (**H**). Relative time spent by BD and control mice in light and dark compartments of light/dark box (**I**). Total time spent in the vicinity of an empty cage vs time spent in the vicinity of a cage with a stranger mouse for BD ($p > 0.05$) and control mice ($p = 0.0041$) (**J**). Difference in grooming duration between BD and control mice during splash test (**K**). Number of activity counts for BD and control mice between 7 and 14 days post FMT (**L**). Percent sucrose preference for BD and control mice between 7 and 14 days post FMT (**M**). BD n = 7, Co n = 7. Data represent mean ± SEM. Significant differences between BD and control groups were analyzed using an unpaired t-test or 2-way ANOVA, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.


A
Aspartic acid
Capric acid
Sucrose
Valeric acid
Fructose
Xanthine
Glucose
Hypoxanthine
-log10(p)
log2(FC)
Status
DOWN
Non-SIG
UP


B
normalized concentration (a.u.)
Co
BD
Butyric acid
Acetic acid
Propionic acid
Succinic acid


C
normalized concentration (a.u.)
Co
BD


D
fold change
Pyy
Gcg

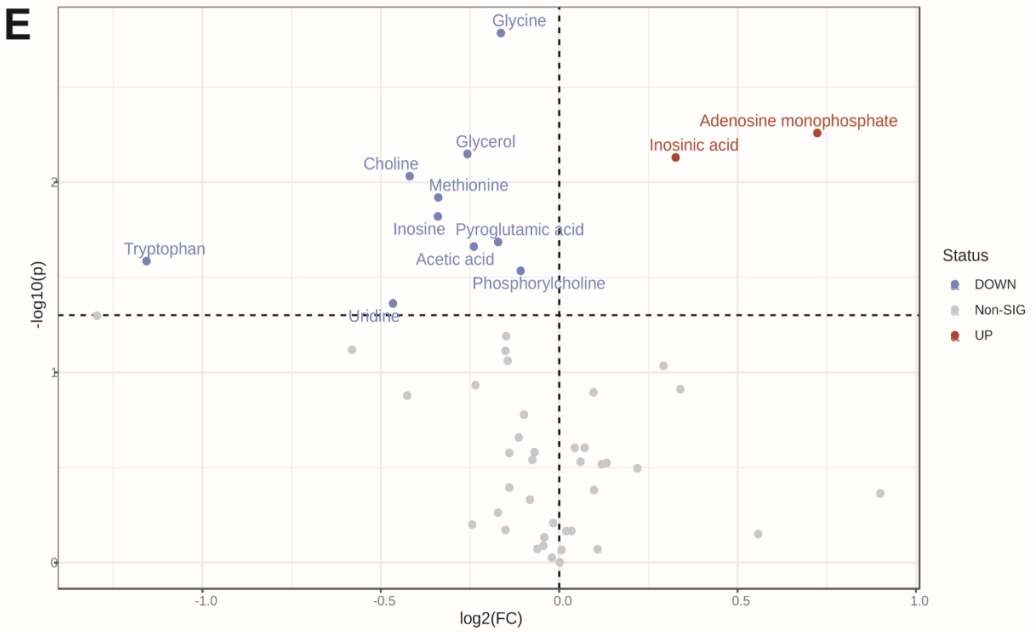

E
Glycine
Adenosine monophosphate
Inosinic acid
Glycerol
Choline
Methionine
Inosine
Pyroglutamic acid
Tryptophan
Acetic acid
Phosphorylcholine
Uridine
-log10(p)
log2(FC)
Status
DOWN
Non-SIG
UP

Fig 3. Fecal and amygdalar metabolite and SCFA profiles of bipolar and healthy control FMT mice. Volcano plot representing the metabolomic profiles of the feces of bipolar (BD) mice plotted relative to control mice 13 days post FMT (**A**). Bar plots with relative levels of short-chain fatty acids acetic acid (2-way ANOVA, $p = 0.017$), butyric acid, propionic acid, and succinic acid in the feces of BD and control groups (**B**). Box plots comparing levels of fecal L-glutamic in BD mice relative to controls (Mann-Whitney, $p = 0.011$). (**C**). Bar plot showing expression of colonic mRNA expression of gut hormones *Pyy* and *Gcg* (2-way ANOVA main effect, $p = 0.003$) in BD and control mice (**D**). Volcano plot representing the metabolomic profiles of BD mice feces plotted relative to control mice (**E**). For both volcano plots, the x-axis represents the log2 fold change (log2(FC)), indicating the magnitude of expression change. The y-axis represents the negative logarithm of the p-value (-log10(p)), indicating the statistical significance of the change. Metabolites with statistically significant upregulation are shown in red, while those with significant downregulation are in blue. Non-significant changes are in gray. The dashed horizontal line depicts the threshold for statistical significance ($p < 0.05$). BD n = 7, Co n = 7. For bar plots, data represent mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{++}p \leq 0.01$.

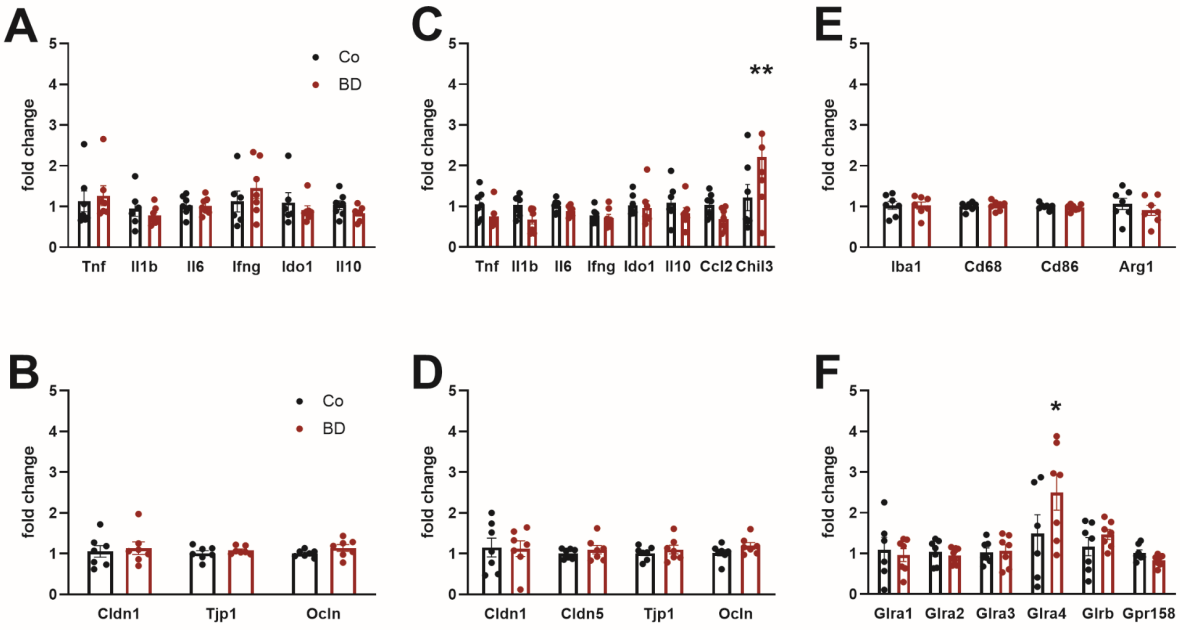


Fig 4. Quantitative polymerase chain reaction (qPCR) determined mRNA expression levels of tight junction proteins, cytokines, and other inflammatory markers in bipolar (BD) and control (Co) FMT mice groups. Bar plot showing that relative colonic mRNA expression of cytokines, inflammatory markers (**A**), and tight junction proteins (**B**) were not significantly different between BD and control mice. Bar plot showing that amygdalar mRNA expression of *Chil3* was significantly up-regulated in BD mice relative to controls ($p = 0.0047$), but expression of other cytokines and inflammatory markers were not differently regulated (**C**). Bar plot showing that tight junction protein expression was similar between BD and control mice (**D**). Bar plot showing that amygdalar mRNA expression levels of glial markers in BD and control mice were not significantly different between BD and control groups (**E**). Bar plot showing that *Glra4* expression was significantly elevated in BD mice ($p = 0.015$), but other glycine receptor subunit proteins were not differentially expressed between groups (**F**). BD n = 7, Co n = 7. Data

represent mean ± SEM. Significant differences between BD and control groups were analyzed using 2-way ANOVA, *$p \leq 0.05$, **$p \leq 0.01$.