The microbial community dynamics of cocaine sensitization in two behaviorally divergent strains of collaborative cross mice

Thi Dong Binh Tran[1], Christian Monroy Hernandez[2], Hoan Nguyen[1], Susan Wright[3], Center for Systems Neurogenetics of Addiction[2], Lisa M. Tarantino[4], Elissa J. Chesler, [2] George M. Weinstock[1], Yanjiao Zhou[5], Jason A. Bubier[‡2]

Running title:

[1]The Jackson Laboratory for Genomic Medicine. 10 Discovery Dive Farmington CT
[2]The Jackson Laboratory for Mammalian Genetics 600 Main St Bar Harbor ME 04605
[3]National Institute of Drug Abuse 6001 Executive Blvd, Rockville, MD 20852.
[4]Department of Genetics, School of Medicine, University of North Carolina, 120 Mason Farm Road, CB7361, Chapel Hill, NC, 27599, USA
[5]School of Medicine, University of Connecticut Health Center, Farmington, CT, United States

Correspondence:

Jason A. Bubier
The Jackson Laboratory
600 Main Street
Bar Harbor ME 04609
Email: Jason.bubier@jax.org

Abstract (250 words)

The gut microbiome-brain axis is increasingly recognized as an important pathway in regulation of cocaine addiction. Microbial products produced in the murine gut have been shown to affect striatal gene expression, and depletion of the microbiome by antibiotic treatment alters cocaine-induced behavioral sensitization in C57BL/6J male mice. Cocaine-induced behavioral sensitization is correlated with drug self-administration behavior, therefore, is a predictor of addiction vulnerability. Here we profile the composition of the naïve microbiome and its response to cocaine sensitization in two Collaborative Cross (CC) strains. These strains display extremely divergent behavioral responses to cocaine sensitization. A high-responding strain, CC004/TauUncJ (CC04), has a gut microbiome that contains a greater amount of *Lactobacillus* than the cocaine-nonresponsive strain CC041/TauUncJ (CC41). The gut microbiome of CC41 is characterized by an abundance of *Eisenbergella, Robinsonella* and *Ruminococcus.* In response to cocaine, CC04 has an increased *Barnsiella* population, while the gut microbiome of CC41 displays no significant changes. PICRUSt functional analysis of the functional potential of the gut microbiome in CC04 shows a significant number of Gut-Brain Modules altered after exposure to cocaine, specifically those encoding for tryptophan synthesis, glutamine metabolism, and menaquinone synthesis (vitamin K2). Depletion of the microbiome by antibiotic treatment revealed a sex-specific altered cocaine-sensitization response to antibiotics in female mice from CC04 that was not observed in male CC04 or either sex of CC41. Together these data suggest a complex relationship between host genetics, microbiome composition and cocaine sensitization behavior.

KEYWORDS

microbiome, cocaine sensitization, collaborative cross, recombinant inbred strains, addiction-related behavior.

FUNDING: U01DA043809 to JAB, GMW P50DA039841 to EJC

1. INTRODUCTION

In the face of the COVID-19 pandemic, the substance use disorder (SUD) and overdose epidemic has continued to grow [1, 2]. Overdose deaths spiked at the start of the pandemic and stayed high throughout 2020 [3]. Among drugs of abuse, cocaine use disorder is highly heritable, with ~70% of the variation ascribed to genetics in twin studies [4]. Advanced genetically diverse mouse strains such as Collaborative Cross (CC) are well suited for exploring the genetic contribution to complex diseases such as cocaine use disorder [5, 6]. Within this population of mice are 45 million SNPs segregating, a level of genetic diversity comparable to that of the human population. The CC mouse population is a panel of recombinant inbred (RI) mice derived from an eight-way cross of distinct sequenced inbred mouse strains. All mice within a CC strain are homozygous and the CC itself is inbred and represents a reproducible population. Approximately 60 CC strains are commercially available. In addition to their diverse phenotypes and host genetics, the microbiome of these recombinant inbred lines exhibits tremendous variation [7, 8]. When controlled for diet and environment, host genetics explains a large part of the variation in microbiome composition [9]. Thus, host genetics may control cocaine use disorder by influencing the microbiome.

The role of the microbiome in SUD is gaining increasing recognition (reviewed in [10, 11]). Previous studies in mice have shown that antibiotic ablation of the microbiome alters low-dose cocaine sensitization and conditioned place preference (CPP) in male C57BL/6J mice [12]. The enhanced CPP response from antibiotics was corrected by short-chain fatty acid administration, suggesting that an intact microbiome 'represses' an endogenous cocaine response. A similar reversible response was also reported for sensitization and CPP in response to morphine in male C57BL/6 mice. [13] Recently treatment of male C57BL/6J mice with Proteobacteria was shown to alter cocaine neurobehavioral responses in a glycine dependent manner[14]. Finally, work in the alcohol field using the binge drinking model, drinking in the dark, using C57BL/6J mice given a 2-week antibiotic pretreatment showed significantly increased alcohol consumption [15]; which this increase could be reversed by sodium butyrate supplementation [16]. However opposite results were obtained in a Wistar derived high drinking selected rat strain in which antibiotic ablation reduced their consumption of ethanol[17]. Ezquer, F., et al restored the high drink phenotype with oral administration of *Lactobacillus rhamnosus,* suggesting the intact microbiome encourages alcohol consumption[17]. Collectively these studies suggest a role for the microbiome in addiction related behaviors.

We examined two previously reported behaviorally divergent CC strains, CC004/TauUnc (CC4) and CC041/TauUnc (CC41) [18]. These strains exhibit extreme genetic and biological variation underlying resistance or vulnerability to the stimulatory and reinforcing effects of cocaine. CC04 mice exhibit a robust locomotor response to cocaine and demonstrate active operant cocaine intravenous self-administration. Conversely, CC41 are non-responsive to the locomotor stimulatory effects of cocaine and are unable to acquire cocaine intravenous self-administration under the same conditions [18]. To determine whether the microbiome contributes to observed cocaine behavior in these CC04 and CC41, we profiled the fecal microbiome before they entered a cocaine sensitization protocol and 19 days later on completion of the protocol by 16S rRNA gene sequencing. We identified distinct microbiome composition and microbiome metabolic pathways in naïve CC04 and CC41 strains, and showed a number of Gut-Brain Modules were altered in the CC04 strain in response to cocaine. Finally, we sought to investigate causation using an antibiotic ablation intervention followed by cocaine sensitization the same strains. In total. we demonstrate that the microbiomes of the CC4 and CC41 strains are unique to each strain and differentially responsive to cocaine. The impact of this work is a demonstration that the role of the microbiome in affecting behavioral response to cocaine is strain specific.

2 MATERIALS AND METHODS

2.1 Animals

The CC004/TauUncJ and CC041/TauUncJ are CC RI strains obtained from The Jackson Laboratory. They were bred in an elevated barrier Pathogen & Opportunistic-Free Animal Room (https://www.jax.org/-/media/jaxweb/health-reports/ax12.pdf?la=en&hash=970E1B297070DD9022BB3CCA38A195F02B4ED295) and transferred at ween to an intermediate barrier specific pathogen-free room (https://www.jax.org/-/media/jaxweb/health-reports/g3b.pdf?la=en&hash=914216EE4F44ADC1585F1EF219CC7F631F881773). All animal protocols were reviewed and approved by The Jackson Laboratory Institutional Animal Care and Use Committee (Approval #10007).

All mice were group-housed with same-sex siblings until six weeks of age in duplex, individually vented cages (Thoren Caging Systems, Inc. Pennsylvania, USA; Cage #3) with pine-shaving bedding (Hancock Lumber) and environmental enrichment consisting of a nestlet and a Shepherd Shack® (Shepard Specialty Papers). Mice were singly housed at six weeks and began behavioral phenotyping at eight weeks of age. Mice were maintained on a 12:12 light cycle (lights on at 6 AM) and allowed *ad libitum* access to the standard rodent chow [sterilized NIH31 5K52 6% fat chow (LabDiet/PMI Nutrition, St. Louis)] and acidified water (pH 2.5–3.0) supplemented with vitamin K. The mice were identified by ear notching at weaning and moved between cages by forceps. Cages were changed once a week. If a cage change was scheduled for a testing day, a clean cage was prepared, and post-test, mice were returned to a new, clean cage. The Jackson Laboratory follows husbandry practices following the recommendations of the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

2.2 Phenotyping

2.2.1 Open Field Apparatus

The open field apparatus was a square-shaped, clear polycarbonate arena (Med-Associates #MED-OFAS-515U) with dimensions 17.5 inches length x 17.5 inches width x10.0 inches height (44.5 cm x 44.5 cm x 25.4 cm). External to the arena's perimeter at the floor level, on the left and right sides, is a pair of horizontal infrared photo beam sensors (16 x 16 beam array). An additional pair of infrared photo beam sensors raised 3 inches from the arena floor (16 x 16 array) are situated at the arena's front and rear outer sides and used to capture vertical activity. Each arena is placed within a sound-attenuating, ventilated cabinet with interior dimensions: 26"W x 20"H x 22"D (Med Associates, #MED-OFA-017) containing a fan that provides white noise. Each cabinet contains two incandescent lights, each affixed in the upper rear two corners of the cabinet at the height of approximately 18.5 inches from the center of the arena floor which provides illumination of 60±10 lux when measured in the center of the arena floor. Data was collected using Activity Monitor software version 7.0.5.10 (SOF-812; Med Associates, Inc; RRID: SCR_014296).

*2.2.*2 Cocaine Sensitization

On all test days, mice were transported from the housing room on a wheeled rack and left undisturbed to acclimate in an anteroom adjacent to the procedure room for a minimum of 30 minutes. Before the first mouse was placed into any behavioral apparatus and between each subject, the equipment was thoroughly sanitized with 70% ethanol solution (in water), and wiped dry with clean paper towels. After all testing for the day, the subjects were returned to the housing room and the arenas were sanitized with Virkon (Lanxess), followed by 70% ethanol to remove any residue. Two different sensitization paradigms were used.

2.2.2.1 Cocaine Sensitization using 10 mg/kg

Complete phenotyping details can be found on the Mouse Phenome Database (https://phenome.jax.org/projects/CSNA03/protocol?method=open+field+test). The cocaine sensitization paradigm was carried out as described in [18]. Briefly, cohorts of mice (3 males, 3 females) of each strain were assigned to sham or cocaine treatment cohorts. Mice were tested for 90 minutes each test day in a 19-day protocol. On days 1, 2 and 12, all animals received saline; on days 3, 5, 7, 8, 11 and 19, half the animals received cocaine (10 mg/kg), while the other half received saline. On each test day, mice were placed in the arena for 30 minutes, then injected with saline or cocaine and returned to the arena for 60 minutes. Before the start of the sensitization paradigm, fresh fecal boli were collected from each mouse. Following the 19-day sensitization paradigm, fecal pellets were collected 60 minutes after the last cocaine injection. Fecal samples were processed and analyzed as described below.

2.2.2.2 Cocaine Sensitization using 5 mg/kg

Following antibiotic treatment (described below), a different cocaine sensitization paradigm was utilized to replicate the protocol of Kiraly 2016 [12]. Briefly, mice were given saline on days one, two and three, and on days 4, 5, 6, 7 and 8, mice were given cocaine at 5 mg/kg i.p. Immediately following cocaine or saline administration, mice were placed in the open field and behavior was monitored for 45 minutes. Numerous behaviors were recorded in the open field as described in Table S1. Before the start of the sensitization paradigm, fresh fecal boli were collected from each mouse. At the completion of testing fecal boli were collected. Fecal samples were processed and analyzed as described below.

2.3 Antibiotic Treatment

Fecal boli samples were collected from treatment-naïve mice. At 8-10 weeks of age, mice started antibiotic treatment (ABX). Mice were administered sulfatrim (19.75 mg/liter sulfamethoxazole + 3.95 mg/liter trimethoprim) and 1 g/L of ampicillin sodium salts, 1g/L of Metronidazole, 0.5 g/L Vancomycin hydrochloride, and 1 g/l of Neomycin (all pharmaceutical grade) in their drinking water continuously. Sweetener (2.5 g/liter, aspartame) was added to the antibiotic water and the water of a second cohort of control (CTRL) mice [7, 19]. One week following the initiation of antibiotic treatment, which was continuous throughout testing, mice from both cohorts were tested in the open field, light-dark and holeboard. Following this baseline testing, 48 mice of both sexes and strains underwent cocaine sensitization in two batches of 24. After testing, fecal boli were harvested from both groups, and cecal contents and striatum were dissected.

2.4 Fecal Collection

Mice were placed in a clean cage for five minutes. Any fecal pellets deposited were collected, placed in Eppendorf tubes, and stored at -80 C. If no pellets were produced in the first five minutes, mice were left in the cage for a longer period.

2.5 Dissections

To control for circadian effects, behavioral testing and euthanasia were consistently performed between 8-12 AM. All surgical instruments were cleaned with RNAase away between each animal (ThermoFischer Scientific). The mice were euthanized by decapitation rather than cervical dislocation in order to maintain the brain structure. The cecum was identified, and the fecal contents were extruded into an Eppendorf tube and flash-frozen on dry ice. All snap-frozen samples were stored at −80°.

2.6 16S Microbiome Analysis

DNA was isolated using the Shoreline complete V1V3 kit (Shoreline Biome, cat #SCV13) per the manufacturer's instructions and used to create barcoded V1V3 amplicons. Briefly, ~5mg of mouse fecal pellet was lysed with a combination of heat, pH, and cell wall disruptors in a single step. DNA was recovered using the magnetic beads, and an aliquot from each sample was transferred to a well in the provided PCR plate containing sample barcode/Illumina sequencing primers. Next, 2x PCR mix from the kit was added, and PCR was performed according to the manufacturer's instructions. After PCR, samples were pooled, purified using a MinElute PCR Purification Kit (Qiagen, cat# 28004) and diluted for sequencing on the Illumina MiSeq (RRID: SCR_020134) platform generating 2X300 paired reads. Sequencing reads are processed by removing the sequences with low quality (average qual <35) and ambiguous codons (N's). Paired amplicon reads are assembled using Flash. Chimeric amplicons were removed using UChime software (RRID: SCR_008057) (73). Our automatic pipeline used the processed reads for operational taxonomic unit (OTU) generation. Each OTU will be classified from phylum to genus level using the most updated RDP classifier and training set (RRID: SCR_006633). A taxonomic abundance table was generated with each row as bacterial taxonomic classification, each column as sample ID and each field with taxonomic abundance. The abundance of a given taxon in a sample was presented as relative abundance (the read counts from a given taxon divided by total reads in the sample)." We also used PICRUSt2 (RRID: SCR_022647) [20] to predict the functional profiling of the bacterial communities by ancestral state reconstruction using 16S rRNA gene sequences. Following the protocol described by Valles-Colomer [21], gut-brain module analysis was performed on the PICRUST2 results. Multiple testing correction of the Gut-Brain Modules was performed using the qvalue package (RRID: SCR_001073).

2.7 Metagenomic whole genome shotgun (mWGS) sequencing, Data Processing and Metagenomics Species Detection

Cecal feces from both cocaine sensitization experiments (with and without antibiotics) were analyzed by mWGS. Libraries are constructed with an average insert size of 500 bases and then sequenced on the HiSeq2500 instrument producing 150 base read pairs from each fragment, yielding ~3 million read pairs/sample. Following mWGS sequencing, sequence data was run through a quality control pipeline to remove poor-quality reads and sequencing artifacts. Sequencing adapters were first removed using TRIMMOMATIC (RRID: SCR_011848) [22]. Next, exact duplicates, low quality, low complexity reads and mouse DNA contamination were removed using GATK-Pathseq (RRID: SCR_005203)[23] pipeline with a k-mer-based approach. For optimization of mouse DNA decontamination, we have built a new GATK-Pathseq 31-mer database by concatenating the following collection of DNA sequences: MM10_GRCm38 reference; sixteen diverse laboratory mouse reference genomes define strain-specific haplotypes and novel functional loci; NCBI UniVec clone vector sequences; repetitive element sequences from RepBase23.02 database; and mouse encode transcripts databases (v25). The final clean reads were used for taxonomic classification and metabolic function analysis for further downstream analysis.

An optimized GATK-Pathseq classification pipeline is time efficient and robust solution for taxonomic classification at the species level. This pipeline used BWA-MEM alignment (minimum 50 bp length at 95% identity). It mapped the final clean reads to the latest updated reference of microbial genomes built by concatenating RefSeq Release 99 (March 2nd, 2020) nucleotide FASTA sequence files of bacteria, viruses, archaea, fungi, and protozoa. Gatk-Pathseq All read counts of microbial species of all kingdoms are used for species abundance analysis.

2.8 Differential abundance analysis of microbial genes and metabolic pathways

The KEGG ortholog (KO) profiling was performed by HumanN2 (RRID: SCR_016280)[24]. Using the DESeq2 package (RRID: SCR_015687)[25] dedicated to performing comparative metagenomics, the inference of the abundance of genes and pathways was obtained and visualized using a volcano plot. Because of the potential high false positive rate of DESeq [26], we plotted raw and relative abundance to inspect the results. Following the protocol described by Valles-Colomer [21], gut-brain module analysis was performed on the mWGS results. Multiple testing correction of the Gut-Brain Modules was performed using the qvalue package (RRID: SCR_001073).

2.9 Biogenic amines analysis

2.9.1 Tissue Extraction

Tissues were frozen at -80º C and shipped to the Vanderbilt Neurochemistry Core for analysis. The samples were held on dry ice prior to the addition of homogenization buffer to prevent the degradation of biogenic amines. Tissues were homogenized using a handheld sonic tissue dismembrator in 100-750 ul of 0.1M TCA containing 0.01M sodium acetate, 0.1mM EDTA, and 10.5 % methanol (pH 3.8). Ten microliters of homogenate were used for the protein assay. The samples were spun in a microcentrifuge at 10,000 g for 20 minutes. Then, the supernatant was removed for HPLC-ECD analysis. HPLC was performed using a Kinetix 2.6um C18 column (4.6 x 100 mm, Phenomenex, Torrance, CA, USA). The same buffer used for tissue homogenization is used as the HPLC mobile phase. The following biogenic amines were analyzed Norepinephrine (NE), 3,4-Dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), serotonin (5-HT),3-methoxytyramine (3-MT).

2.9.2 Protein assay

Protein concentration in cell pellets was determined by BCA Protein Assay Kit (Thermo Scientific). Ten microliter tissue homogenate is distributed into a 96-well plate and 200 μl) mixed BCA reagent (25 ml of Protein Reagent A is mixed with 500 μl of Protein Reagent B) is added. Incubate the plate at room temperature for two hours for color development. A BSA standard curve is run at the same time. Absorbance was measured by the plate reader (POLARstar Omega), purchased from BMG LABTECH Company.

2.10 Statistics

Microbial community analysis was performed by R version 3.5.1 (RRID: SCR_001905). Principal coordinate analysis (PCoA) plots, boxplots and heatmaps were generated for graphical visualization using Phyloseq, ggplot2 (RRID: SCR_014601)[27] and ComplexHeatmap (RRID: SCR_017270)[28] packages. Richness was calculated as the number of OTUs present in each sample. The Shannon Diversity Index combined species richness, and the evenness was computed as $\Sigma p_i * \ln(p_i)$, where pi presents the proportional abundance of species. The non-parametric Wilcoxon or Kruskal-Wallis rank sum-tests were used for differential diversity or abundance between two or more groups and corrected for multiple comparisons by the Benjamini-Hochberg procedure. Beta diversity was analyzed at the OTU level using the Bray-Curtis distance for community abundance and the Jaccard distance for community presence/absence.

The among-group differences were determined using the permutational multivariate analysis of variance by the distance matrices (ADONIS). These tests compare the intragroup distances to the intergroup distances in a permutation scheme and then calculate a p-value. These functions are implemented in the Vegan package (RRID: SCR_011950)[29]. For all permutation tests, we used 10,000 permutations.

Statistical analyses for sensitization and neuropeptides (sensitization only) were conducted using JMP 16 (SAS Institute; RRID: SCR_014242). The best model is

$$\text{Phenotype}=\beta_0\text{Sex}+\beta_1\text{Genotype}+\beta_2\text{Treatment}+\beta_2(\text{Sex}\times\text{Genotype}\times\text{Treatment})+\beta_3(\text{Sex}\times\text{Genotype})+\beta 4(\text{Sex}\times\text{Treatment})+\beta 5\,(\text{Treatment}\times\text{Genotype})+\underset{\sim}{U}+\varepsilon,$$

where ε is random error. The parameters (β, ε) were estimated by Type III non-sequential ordinary least squares in the ANOVA model. Repeated measures analysis of variance was performed using MANOVA to estimate the effects of strain, sex, treatment and their interaction. $\underset{\sim}{U}$ represents the symmetric nature of the variance co-variance matrix of the random effects. In all cases, the full model was fit and reduced by dropping non-significant inter-actions followed by main effects. In order to determine the nature of differences detected in the ANOVA model, planned contrasts were performed giving the terms not included in the model a weight of zero and giving the terms to be compared (treatment, strain) values of opposite weights, −1 and +1.

3 Results

3.1 Fecal microbiome composition of CC004 and CC041 lines

We sought to determine the fecal microbiome composition of two strains of CC mice shown to be behaviorally divergent in response to cocaine[18]. We performed 16S V1-V3 sequencing on fecal boli from naïve male and female mice and mice that had completed a 19-day cocaine sensitization paradigm. A principal coordinates analysis of the beta-diversity of the gut microbiome showed significant separation of the microbiomes by strain (PC1 35.8%) and cocaine effect (PC2 13.4%) (**Figure 1A**). Analysis of Group Dissimilarity-Adonis based on microbial abundance matrix with Bray-Curtis distance showed a significant difference between the naïve and cocaine exposure groups in the CC04, [Adonis $F_{(3,22)}$ = 4.3768 p <0.001, R2=0.40866]. This cocaine effect was strain specific [CC04 $F_{(1,10)}$ =2.6019, p<0.05 R2=0.186; CC41 $F_{(1,11)}$ =1.2826, p=0.17 R^2=0.011]

Naïve CC04 displayed decreased *Eisenbergella* (W=9, Z=-3.80, q<0.0001 r=0.79), *Aneroplasma* (W=0, Z=-4.27, q<0.001, r=0.89), *Robinsonella* (W=23 Z=-2.69 q=0.018, r=0.56), and *Ruminococcus* (W=5.5, Z=-3.98, q<0.001, r=0.83) and an increase in *Lactobacillus* (W=126, Z=-3.69, q=0.001, r=0.77), and *Anaerostipes* (W=128, Z=-3.90, q <0.0001, r=0.813), compared to Naive CC41. (**Figure 1B-D**). In response to cocaine, CC04 showed an increase in *Barnsiella* (W=0, Z=-3.63, q=0.0082, r=0.93) and *unclassified_Coriobacteriaceae* (W=0, Z=-3.60, q<0.0082, r=0.93). CC41 showed no significant differences that passed a stringent FDR=0.05 after cocaine treatment. *Barnsiella* did show an increase (W=20.5, Z=-1.92, p=0.05, q=0.34, r=0.45) in CC41 but it did not pass multiple correction testing. A full table of differences, (p<0.05 and q>0.05) between strains can be found in **Table S2.**

3.2 PICRUSt analysis of the 16S data identifies molecular functions that differ between strains and those that are affected by cocaine treatment in CC04 mice.

Based upon the linkage between phylogeny and function, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) is an approach to predictive metagenomics that has been demonstrated to provide useful insights into thousands of uncultivated microbial communities [30]. PICRUSt was used to compare the functional properties of the microbiome before the sensitization paradigm with the microbiome after the sensitization paradigm in both CC04 and CC41 mice. Outlier samples (19183 CC04 pre and 19179 CC04 Post) were removed for this analysis. These samples appeared to be switched due to their position in the PCA, although results were similar when they were included. There were 50 Gut-Brain KEGG Modules that were significantly different, after correcting for multiple testing, between naïve CC04 and naïve CC41. (**Table S3**, **Figure 2A,B**). The KO0686 other glutamine showed greatest upregulation (4.48 of log2 fold change) in CC04 mice, compared to CC41 mice. log fold change (LFC) of 4.48, and the most downregulated was KO12942 other_glutamate LFC -6.08. Other modules represented multiple times included Menaquinone synthesis (vitamin K2), Isovaleric acid synthesis I (KADH pathway), acetate and propionate metabolism. The KEGG ontology pathways, represented by CC04 microbiome post-sensitization, included 29 Gut-Brain KEGG Modules that were significantly different after correcting for multiple testing **(Table S3, Figure 2C,D**). There were three downregulated modules related to tryptophan synthesis, four downregulated modeules related to glutamine metabolism, seven modules related to Menaquinone synthesis (vitamin K2), and others related to glutamate and GABA synthesis. The greatest fold change was -7.06 LFC for other_glutamine K12942 (q=0.007).

3.3 Antibiotic treatment effects on cocaine sensitization behavior

Previous work has shown the involvement of the microbiome in cocaine sensitization and CPP in male C57BL/6J mice at low doses of cocaine (5 mg/kg) [12]. CC04 and CC41 mice were given *ad libitum* access to antibiotic-treated water or control (aspartame) water starting at 8-10 weeks of age (**Figure 3**) to test for the involvement of the microbiome in observed differences in cocaine sensitization response in these two strains. The antibiotic treatment didn't cause any adverse effect on the mice as measured by weight differences between the control and treated groups (**Figure S1**). In a repeated-measures ANOVA of the full factorial model that was sequentially reduced there were no significant strain × sex × treatment effects or interactions and only a univariate effect of sex was observed ($F_{(1,22)}$=16.171 p=0.0006). The CC04 and CC41 strains were behaviorally divergent, as previously published, with CC04 male and female mice responding to cocaine and the CC41 mice being unresponsive to the drug (**Figure 4).** One mouse was excluded as a behavioral outlier (male, CC04, 25760 ABX). Pairwise t-tests at each time point show no significant difference between treated

and control mice. However, a repeated measures ANOVA on each sex of mice showed that in females there was a time x strain x treatment effect [$F_{(7,13)}$ = 1.692784 = p = 0.0356] Post-hoc contrast analysis showed CC04 antibiotics vs control $F_{(7,13)}$= 3.64, p= 0.0156; whereas CC41 antibiotics vs control $F_{(7,13)}$=0.01318, p = 1. Univariate effect of strain $F_{(1,10)}$ = 70.874, p <0.001. However in males, there was a univariate effect of strain $F_{(1,18)}$ = 38.4406, p<0.001 but not a significant time × strain × treatment $F=_{(7,12)}$ = 0.630, p = 0.4407.

3.4 Effect of antibiotic treatment on the microbiome of CC strains that vary in response to cocaine
16S and mWGS were performed on the **cecal** contents on all mice at the end of the cocaine sensitization paradigm. ABX treatment significantly reduced the bacterial abundance in both the CC04 and CC41 strains as seen in WGS read counts (**Figure S2**). Consistent with fecal microbiome data, CC04 showed a higher abundance of *Barnsiella* (W=30, Z=-2.85, p=0.004, q=0.04, r=.86) post sensitization in comparison to CC41 post sensitization in the cecal microbiome. CC41 showed a higher abundance of *Eisenbergiella* (W=3, Z=-2.15, p=0.03, q=0.1) compared to CC04 post sensitization (**Figure 5, Table S4**). No comparisons were made between ABX samples as the reads were too low due to the efficacy of the antibiotic ablation. mWGS of cecal contents obtained from mice post cocaine sensitization showed an abundance of *Lactobacillus johnsonii* (W=30, Z=-2.85 p=0.004, q=0.26, r=0.86) and *Muribaculum intestinale* (W=30, Z=-2.83, p=0.004, q=0.26, r=0.86) in CC04. *Akkermansia muciniphila* (W=3, Z=-2.166, p=0.03, q=0.48, r=.65) were dominant in CC41 mice. *Duncaniella* abundance was also different (W=30, Z=-2.85, p=-0.004, q=0.26) with higher levels found in CC04 (**Figure 5B, Table S4).** Different results obtained by 16S versus mWGS data profiling are to be expected based upon the type of reference database available for each technique. To produce more comparable data, 16S data was mapped to the PathSeq database used for analyzing WGS data. In this analysis *Lactobacillus* and *Akkermansia* trends remained the same (**Figure S3**).

3.5 PICRUSt analysis of the cecal microbiome of 16S data identifies differential KO modules between CC04 and CC41 strains after cocaine treatment. We have previously observed KO cluster differences in CC04 in response to cocaine that were absent in the CC41 strain. Based upon PICRUSt analysis using control samples, 68 KO modules were significantly different across the CTRL strains cocaine-sensitization treatment (**Figure 6A,B Table S5).** Of those KO modules 20 were associated with GABA/glutamine/glutamate metabolism and seven related to short-chain fatty acid metabolism (acetate, butyrate, propionate). The KO modules K11102 other_glutamate and K13923 Propionate synthesis had the largest fold change (LFC -5.1) between the strains.

3.6 Pathway analysis using mWGS cecum data
Utilizing the mWGS samples, we looked at the representation of the GBM pathways in the control CC4 vs CC41 samples post cocaine sensitization protocol. 13 pathways were down regulated and 14 up regulated in CC04 compared to CC41 mice (**Figure 7 and Table S6**). PWY.4321.L.glutamate.degradation.IV (LFD -3.317, p=0.0001, q=0.001) was more abundant in CC04, as was PWY.5044 purine nucleotide degradation (LFC=1.78, p=0.006, q=0.025) and PWY6666.2.dopamine.degradation (LFC -4.943, p=0.016, q=0.05).

3.7 Antibiotic treatment effects on striatal neurotransmitter levels.
We observed a significant difference in DOPAC (p<0.0001), HVA (p<0.001), 3-MT (p=0.0077) and DA (p=0.008) between CC004 and CC0041 strains before antibiotic treatment. There was no significant effect of strain by treatment, except for DA levels. For DA, the full model there was a strain × treatment effect $F_{(3,41)}$=4.4089, p=0.0093. In a post hoc contrast of the control group there was a significant difference between CC04 vs CC041. However in the antibiotic group there was not a significant difference $F_{(1,38)}$=4.0438, p=0.0515. In summary, strain differences in striatal dopamine were diminished by antibiotic treatment (**Table 1**).

4. DISCUSSION±

Extensive studies have shown that host genetics have a causal role in the structure and composition of the gut microbiome [8, 9]. We have previously identified QTL regulating the abundance of numerous bacteria in the CC breeding population [7], and demonstrate here how two behaviorally divergent CC strains [18] also have unique microbiome compositions. We have confirmed that repeated cocaine treatment of male and female CC04 mice results in an upregulation of *Barnsiella* as was reported in the colon of male C57BL/6N mice [31]. We have also shown that the effects of the cocaine sensitization paradigm upon the microbiome are strain

specific and that an intact microbiome (not antibiotic treated) does not significantly repress a low dose cocaine sensitization response as observed in C57BL/6J males [12]. We did observe that naïve strain differences in striatal DOPAC between CC04 and CC41 are abolished by antibiotic treatment. These findings are consistent with host genetics controlling both the differential behavior observed in these two strains, their innate microbiome composition, and the microbiome's response to repeated drug exposure.

CC strains have been observed to express a great deal of variation in addiction and addiction-related traits[18, 32-34]. Phenotyping this reference panel of RI lines has revealed phenotypic variation much more extreme than originally observed in the eight inbred founder strains. The microbial differences include numerous taxa such as *Acetatifactor, Amerpfustis, Anaeroplasma, Butyrivibrio, Catabacter, Christensenella, Eisenbergiella, Enterococcus, Klebsiella, Lactobacillus, Parvibacter, Robinsoniella, Roseburia, Ruminococcus, Syntrophococcus* and several unclassified microbes among the 82 most abundant genera.

One of the surprising results of this study was the observation that antibiotic treatment did not dramatically increase behavioral sensitization to a dose of 5 mg/kg cocaine in male and female mice of the CC04 and CC41 strains. Instead, we observed a sex-specific response to antibiotics in CC04 female mice that was not observed in CC04 males or either sex of CC41. While the published data showed differences in cocaine sensitization behavior at 5 mg/kg, there were no behavioral differences of antibiotic treatment at 10 mg/kg in the male B6J mice tested in that same publication[12]. This suggested that the repressive effect of the microbiome on sensitization behavior may be dose dependent. In our antibiotic experiment, we tested to a single dose of cocaine (5mg/kg) and perhaps CC04, a very high responder would have required a lower dose of cocaine to see antibiotics able to remove the repressive effect of the microbiome on sensitization behavior. Similarly, CC41 mice exhibit no locomotor response to cocaine at the published dose of 10mg/kg [18] and 5 mg/kg might have shown a more dramatic behavioral response at a higher concentration of cocaine, that could be altered by the microbiome. Our results, together with those of Kiraly suggest that the microbiome effect on locomotor sensitization in response to cocaine is dependent on strain, sex and dose of cocaine used [12].

One of the most dramatic differences is the abundance of *Eisenbergiella* in CC41, a strain that shows little locomotor response to cocaine and an increase in *Lactobacillus* in CC04 strain that is highly responsive to the locomotor effects of cocaine. It is interesting to note that the strain that is not behaviorally responsive to cocaine did not have any microbiota that were significantly different before and after cocaine administration, which is in stark comparison to CC04 which is very responsive and exhibited a large increase in *Barnesiella* and smaller increase in *unclassified_Coriobacteriaceae*. Previously a study that treated C57BL/6N male mice with 20 mg/kg of cocaine ip for 7 consecutive days identified four genera that were decreased, *Mucispirillum, Butyricicoccus*, *Pseudoflavonifractor* and unclassified *Ruminococcaceae* while there was an increase in *Barnesiella*, and unclassified members of *Porphyromonadaceae*, *Bacteriodales*, and *Proteobacteria* [31]. It must be noted that the C57BL/6N strain of mouse in comparison to the C57BL/6J strain of mice contains a mutation in *Cyfip2* gene and presents with a relatively dampened cocaine sensitization response [35]. The authors suggest that the change in microbiome composition, dysbiosis, was associated with upregulation of proinflammatory mediators including NF-κB and IL-1β. Similarly, they show that cocaine altered the gut-barrier composition of the tight-junction resulting in a leaky gut. While the authors used only one sex, one strain and one high repeated dose of cocaine, we have used multiple strains and see the dysbiosis phenotype only in the strain that is behaviorally responsive to both doses of cocaine [12].

The differential metabolic potential that was identified using either PICRUSt or mWGS pointed to both glutamate degradation and dopamine degradation enzymes encoded by the microbiome as upregulated in CC04 but not CC41 in response to cocaine. Glutamate receptors line the epithelial cells of the gut and have been show to signal through vagal afferents to regions of the brain [36, 37]. Glutamate is also the substrate for GABA production and GABA producing strains such as *Lactococcus lactis* have been shown to modulate behavior [38]. The fining that glutamate metabolism is altered in response to cocaine in responsive strains supports the glutamate hypothesis of addiction [39-41], but now extends that process into the gut. These observations provide some mechanistic insight into the gut-brain axis and SUD.

5. CONCLUSION

In conclusion, our study indicates that the microbiome shows changes in response to cocaine, in a strain dependent manner. Strains such as CC041 that appear behaviorally non-responsive to cocaine, also contain a microbiome that does not robustly respond to cocaine. However, strain CC04 that exhibits a strong locomotor response to cocaine also displays dramatic changes with its microbiome composition in response to both a high and low dose of cocaine. These changes in response to cocaine are detectable at the level of changes to the metabolic potential of the microbiome. CC04 has an increase in microbes with genomes containing an abundance of glutamate and dopamine metabolic pathways. Furthermore, antibiotic ablation of the microbiome does not alter the sensitization response of either strain to 5 mg/kg cocaine - CC41 mice remain non-responsive and CC04 females showed only a minimal increase in locomotor activity in response to antibiotics.

Figure legends.

Figure 1- Microbial composition of two naïve Collaborative Cross strains and in response to cocaine sensitization paradigm. A. Principal components analysis of bacterial beta diversity at OTU level using Bray-Curtis for 16S microbiome data showing clustering of samples by host genotype (PC1 35.8%) and Treatment (PC2 13.4%). Three males and three females of each strain were tested with cocaine or a sham treatment. **B**. The percent abundance of the 20 most abundant genera in female and male mice of strain CC04 and CC041 before a cocaine sensitization paradigm and after a cocaine sensitization paradigm. **C.** Strain and Treatment average microbiome composition of the top 20 genera. **D.** Heat map representation of the microbiome composition of CC04 and CC041 pre and post cocaine sensitization.

Figure 2 Volcano plots of the functional categories encoded by the microbiome. A. A scatterplot showing the statistical significance and magnitude of change of the KO clusters as determined by PICRUSt2 of 16S data between CC04 and CC041 pre sensitization**.** Blue=qed >0.05, Red=qed <0.05**. B.** The gut-brain module KO categories (qed<0.05) from PICRUSt2 of 16S data. **C**. A scatterplot showing the statistical significance and **D**. magnitude of change of the gut-brain module KO clusters as determined by PICRUSt2 of 16S data between CC04 pre sensitization and CC04 post sensitization. *****Outlier samples 19183 CC4 pre and 19179 CC4 Post were removed from the later PICRUSt analysis.

Figure 3 Schematic diagram of the experimental design for Antibiotic ablation of the microbiome of CC4 and CC41 mice.

Figure 4-Cocaine behavioral sensitization of male and female CC04 and CC041 mice in response to antibiotic treatment and with control. The behavioral sensitization of Kiraly [12] was followed which included three days of saline treatment followed by five days of 5 mg/kg i.p. cocaine administration.

Figure 5- Microbiome composition of CC04 and CC41 cecal contents by 16S and Whole Genome Shotgun sequencing of control mice following cocaine sensitization. Differences in *Lactobacillus* and *Akkermansia* trend similarly in both methods of microbiome analysis.

Figure 6- Volcano plots of the functional categories encoded by the microbiome 16S PICRUSt and the modules that are differential between strains after cocaine sensitization. A. A scatterplot showing the statistical significance and magnitude of change of the KO clusters as determined by PICRUSt2 of 16S data**. B.** The gut-brain module KO categories (qed<0.05) from PICRUSt2 of 16S data

Figure 7- The WGS pathways that are differential in the cecal feces between CC4 and CC41 post sensitization. A. A scatterplot showing the statistical significance and magnitude of change of the KO pathways from WGS sequencing. **B.** Example pathways differential representation across CC4 and CC41 mice after cocaine sensitization.) Blue=significant KO/pathway (p adjusted value<0.05), red=significant KOs that are GBM (p adjusted value<0.05).

SUPPORTING INFORATION

Table S1 The variable names and all the metadata generated by open field

Table S2- Tab 1-Differences between CC04 and CC41.Tab 2- Differences between CC4 in response to Cocaine. Tab 3- Differences between CC41 in response to Cocaine. Pink cells represent p<0.05.

Table S3- Tab 1- Gut-Brain Modules from PICRUSt that differed (p<0.05) between CC04 and CC41 Tab 2- Gut-Brain Modules from PICRUSt that differed (p<0.05) between CC04 naïve and CC04 post Tarantino Cocaine Sensitization.

Table S4- Tab 1 Differences between cecum of CC04 and CC41 post cocaine sensitization paradigm of Kiraly. Tab 2 WGS Differences between cecum of CC04 and CC41 post cocaine sensitization paradigm of Kiraly.

Table S5 Gut-Brain Modules from PICRUSt of 16S data that differed (p<0.05) between CC04 and CC41 in cecum samples.

Table S6 KEGG Orthology pathways that were differentially abundant in the cecum of CC4 vs CC41 mice based upon the functional potential of the WGS data.

Figure S1 Weight of animals over the eight days of the cocaine sensitization protocol. Based upon weight there was no adverse effect of the antibiotic treatment upon animal weight.

Figure S2 Total read counts from the control and antibiotic treated mice. There were very few reads recovered from the antibiotic treated mice, which displayed enlarged cecums consistent with a severe microbiome disruption.

Figure S3. **Microbiome composition of CC04 and CC41 cecal contents by 16S and Whole Genome Shotgun sequencing of control mice following cocaine sensitization** For this analysis the 16S data was mapped to Pathseq complete genome (90% similarity).

Table 1- The mean and standard error of various neurotransmitters in the striatum of CC04 and CC041 mice with out without antibiotic treatment. NE=Norepinephrine, DOPAC=3,4-Dihydroxyphenylacetic acid, DA=dopamine, 5-HIAA= 5-hydroxyindoleacetic acid, HVA= homovanillic acid, 5-HT=serotonin, 3-MT= 3-methoxytyramine. CTRL=Control treatment water with aspartame sweetener. ABX=triple antibiotic cocktail with aspartame sweetener.** <0.01 Measurements are reported as ng/mg total protein.

Strain	Treatment	NE	DOPAC	DA	5-HIAA	HVA	5-HT	3-MT
CC04/TauUncJ n=10	CTRL	4.1 ± 0.6	7.7 ± 0.5**	164.3 ± 8.2**	4.8 ± 0.2	14.4 ± 0.7**	16.2 ± 0.7	14.3 ± 0.7**
CC41/TauUncJ n=11	CTRL	3.4 ± 0.5	11.3 ± 0.5	203.9 ± 6.1	5.2 ± 0.3	17.9 ± 0.5	17.9 ± 1.2	12.2 ± 0.4
CC04/TauUncJ n=11	ABX	4.2 ± 0.5	9.0 ± 0.7**	169.8 ± 5.8	4.9 ± 0.2	13.9 ± 0.6**	17.2 ± 1.0	15.0 ± 0.7**
CC41/TauUncJ n=10	ABX	3.6 ± 0.3	11.5 ± 1.0	196.2 ± 15	5.4 ± 0.4	16.8 ± 1.0	19.1 ± 1.1	13.0 ± 1.1

ACKNOWLEDGEMENT

The authors gratefully acknowledge drug reagents from the NIDA Drug Supply Program. The Vanderbilt Neurochemistry Core is supported by the Vanderbilt University Brain Institute.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest

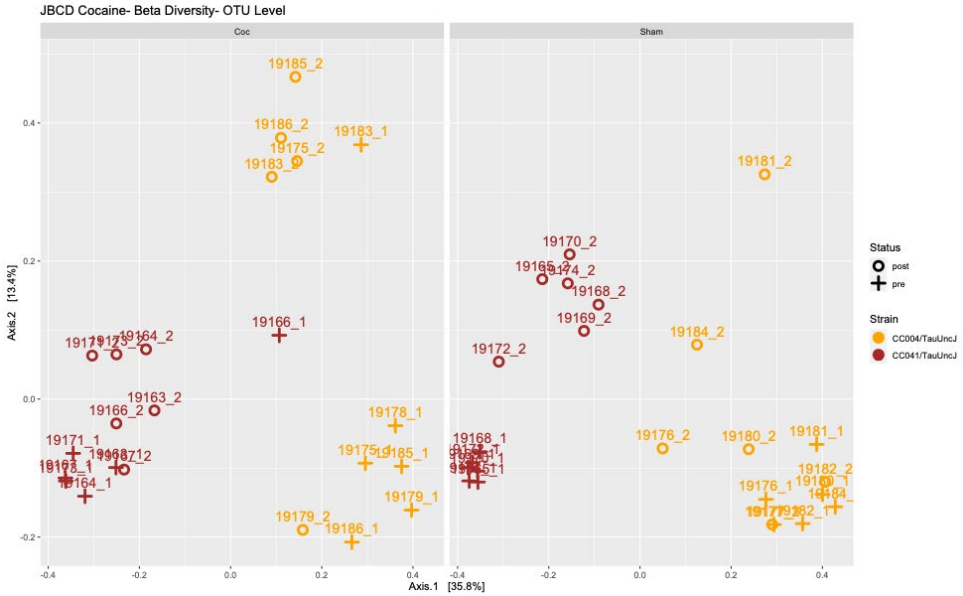
DATA AVAILABILITY STATEMENT

The 16S and WGS data are available on SRA under accession SUB12204043.

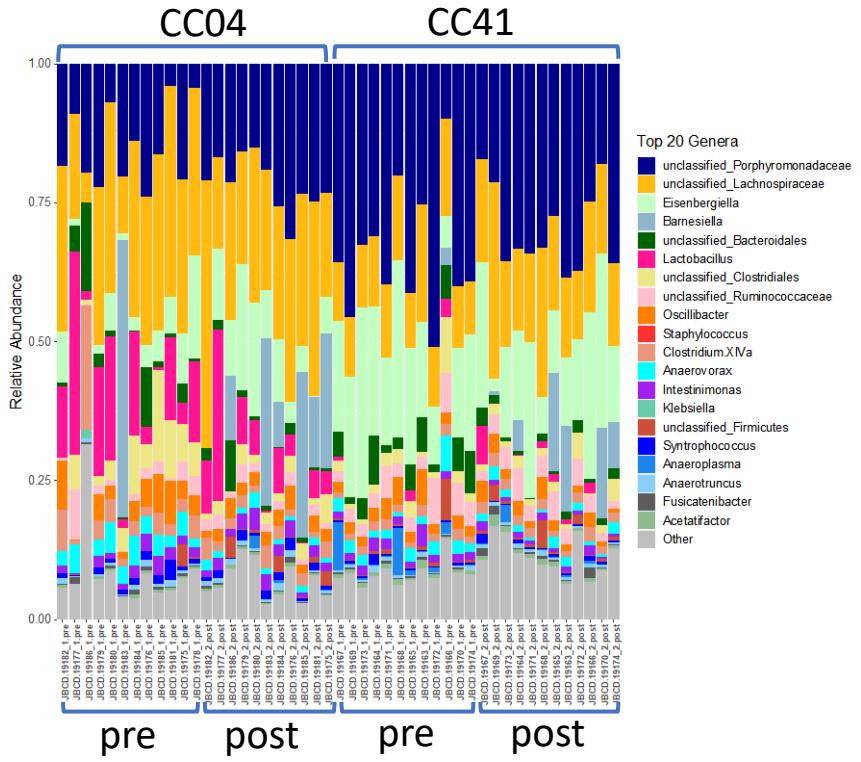
1. Niles, J.K., et al., *The Opioid Epidemic Within the COVID-19 Pandemic: Drug Testing in 2020.* Popul Health Manag, 2021. **24**(S1): p. S43-S51.
2. Wang, Q.Q., et al., *COVID-19 risk and outcomes in patients with substance use disorders: analyses from electronic health records in the United States.* Mol Psychiatry, 2021. **26**(1): p. 30-39.
3. Ahmad, F., A. Rossen, and P. Sutton. *Provisional drug overdose death counts.* 2021 [cited 2021 9/9/2021]; Available from: https://www.cdc.gov/nchs/nvss/vsrr/drug-overdose-data.htm.
4. Goldman, D., G. Oroszi, and F. Ducci, *The genetics of addictions: uncovering the genes.* Nat Rev Genet, 2005. **6**(7): p. 521-32.
5. Logan, R.W., et al., *High-precision genetic mapping of behavioral traits in the diversity outbred mouse population.* Genes Brain Behav, 2013. **12**(4): p. 424-37.
6. Saul, M.C., et al., *High-Diversity Mouse Populations for Complex Traits.* Trends Genet, 2019. **35**(7): p. 501-514.
7. Bubier, J.A., et al., *A Microbe Associated with Sleep Revealed by a Novel Systems Genetic Analysis of the Microbiome in Collaborative Cross Mice.* Genetics, 2020. **214**(3): p. 719-733.
8. Campbell, J.H., et al., *Host genetic and environmental effects on mouse intestinal microbiota.* ISME J, 2012. **6**(11): p. 2033-44.
9. Bubier, J.A., E.J. Chesler, and G.M. Weinstock, *Host genetic control of gut microbiome composition.* Mamm Genome, 2021. **32**(4): p. 263-281.
10. Russell, J.T., et al., *The Gut Microbiome and Substance Use Disorder.* Front Neurosci, 2021. **15**: p. 725500.
11. Chivero, E.T., et al., *Substance use, microbiome and psychiatric disorders.* Pharmacol Biochem Behav, 2022. **219**: p. 173432.
12. Kiraly, D.D., et al., *Alterations of the Host Microbiome Affect Behavioral Responses to Cocaine.* Sci Rep, 2016. **6**: p. 35455.
13. Hofford, R.S., et al., *Alterations in microbiome composition and metabolic byproducts drive behavioral and transcriptional responses to morphine.* Neuropsychopharmacology, 2021.
14. Cuesta, S., et al., *Gut colonization by Proteobacteria alters host metabolism and modulates cocaine neurobehavioral responses.* Cell Host Microbe, 2022. **30**(11): p. 1615-1629 e5.
15. Reyes, R.E.N., et al., *Antibiotic-induced disruption of commensal microbiome linked to increases in binge-like ethanol consumption behavior.* Brain Res, 2020. **1747**: p. 147067.
16. Reyes, R.E., et al., *Supplementation with sodium butyrate protects against antibiotic-induced increases in ethanol consumption behavior in mice.* Alcohol, 2022. **100**: p. 1-9.
17. Ezquer, F., et al., *Innate gut microbiota predisposes to high alcohol consumption.* Addict Biol, 2021. **26**(4): p. e13018.
18. Schoenrock, S.A., et al., *Characterization of genetically complex Collaborative Cross mouse strains that model divergent locomotor activating and reinforcing properties of cocaine.* Psychopharmacology (Berl), 2020. **237**(4): p. 979-996.
19. Wu, H.J., et al., *Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells.* Immunity, 2010. **32**(6): p. 815-27.
20. Douglas, G.M., et al., *PICRUSt2 for prediction of metagenome functions.* Nat Biotechnol, 2020. **38**(6): p. 685-688.
21. Valles-Colomer, M., et al., *The neuroactive potential of the human gut microbiota in quality of life and depression.* Nat Microbiol, 2019. **4**(4): p. 623-632.
22. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data.* Bioinformatics, 2014. **30**(15): p. 2114-20.
23. Walker, M.A., et al., *GATK PathSeq: a customizable computational tool for the discovery and identification of microbial sequences in libraries from eukaryotic hosts.* Bioinformatics, 2018. **34**(24): p. 4287-4289.
24. Franzosa, E.A., et al., *Species-level functional profiling of metagenomes and metatranscriptomes.* Nat Methods, 2018. **15**(11): p. 962-968.

25. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biol, 2014. **15**(12): p. 550.
26. Weiss, S., et al., *Normalization and microbial differential abundance strategies depend upon data characteristics.* Microbiome, 2017. **5**(1): p. 27.
27. Wickham, H., *ggplot2 : Elegant Graphics for Data Analysis*, in *Use R!*,. 2016, Springer International Publishing : Imprint: Springer,: Cham. p. 1 online resource (XVI, 260 pages 232 illustrations, 140 illustrations in color.
28. Gu, Z., R. Eils, and M. Schlesner, *Complex heatmaps reveal patterns and correlations in multidimensional genomic data.* Bioinformatics, 2016. **32**(18): p. 2847-9.
29. Jari Oksanen, F.G.B., Michael Friendly, Roeland Kindt,Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B. O'Hara,Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs,Helene Wagner, *Vegan Package.* 2020: p. 298.
30. Langille, M.G., et al., *Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences.* Nat Biotechnol, 2013. **31**(9): p. 814-21.
31. Chivero, E.T., et al., *Cocaine Induces Inflammatory Gut Milieu by Compromising the Mucosal Barrier Integrity and Altering the Gut Microbiota Colonization.* Sci Rep, 2019. **9**(1): p. 12187.
32. Bailey, L.S., et al., *Repeated dosing with cocaine produces strain-dependent effects on responding for conditioned reinforcement in Collaborative Cross mice.* Psychopharmacology (Berl), 2022.
33. Bagley, J.R., et al., *Extreme phenotypic diversity in operant response to intravenous cocaine or saline infusion in the hybrid mouse diversity panel.* Addict Biol, 2022. **27**(3): p. e13162.
34. Bailey, L.S., et al., *Heritable variation in locomotion, reward sensitivity and impulsive behaviors in a genetically diverse inbred mouse panel.* Genes Brain Behav, 2021. **20**(8): p. e12773.
35. Kumar, V., et al., *C57BL/6N mutation in cytoplasmic FMRP interacting protein 2 regulates cocaine response.* Science, 2013. **342**(6165): p. 1508-12.
36. Kondoh, T., H.N. Mallick, and K. Torii, *Activation of the gut-brain axis by dietary glutamate and physiologic significance in energy homeostasis.* Am J Clin Nutr, 2009. **90**(3): p. 832S-837S.
37. Furness, J.B., et al., *The enteric nervous system and gastrointestinal innervation: integrated local and central control.* Adv Exp Med Biol, 2014. **817**: p. 39-71.
38. Gao, K., et al., *Oral administration of Lactococcus lactis WHH2078 alleviates depressive and anxiety symptoms in mice with induced chronic stress.* Food Funct, 2022. **13**(2): p. 957-969.
39. Kalivas, P.W., *The glutamate homeostasis hypothesis of addiction.* Nat Rev Neurosci, 2009. **10**(8): p. 561-72.
40. Koob, G.F. and E.J. Nestler, *The neurobiology of drug addiction.* J Neuropsychiatry Clin Neurosci, 1997. **9**(3): p. 482-97.
41. Koob, G.F. and N.D. Volkow, *Neurobiology of addiction: a neurocircuitry analysis.* Lancet Psychiatry, 2016. **3**(8): p. 760-773.

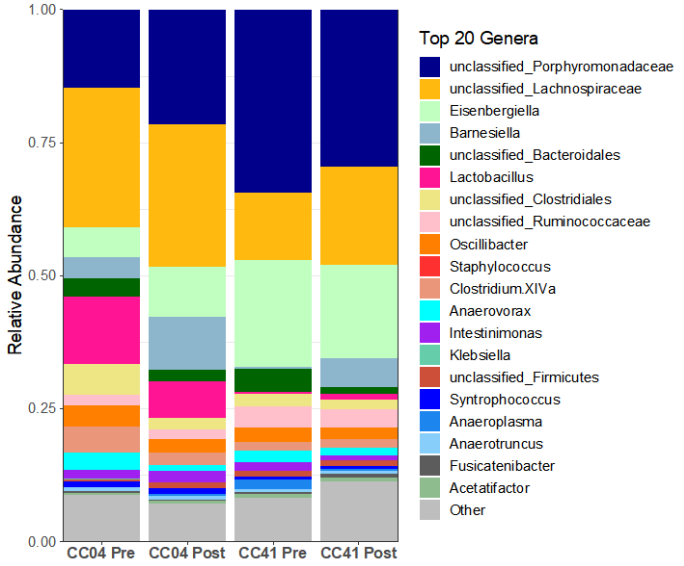
A



B



C



D

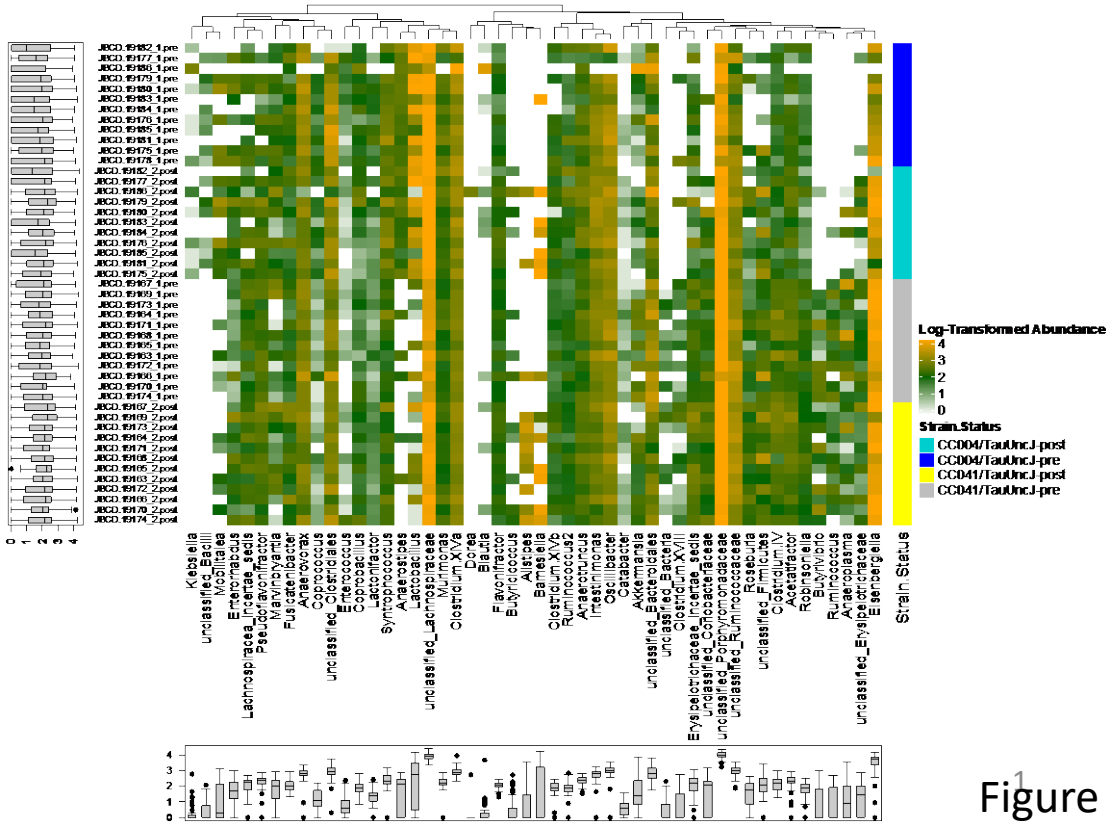
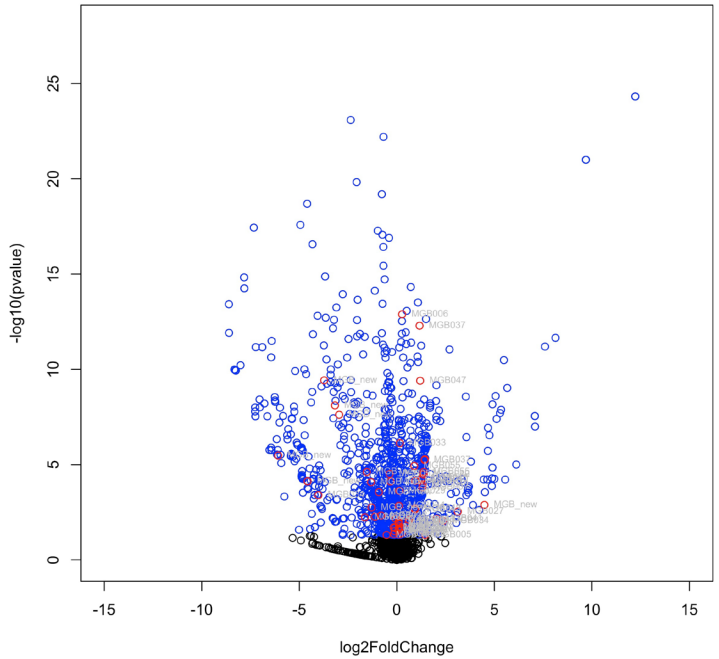


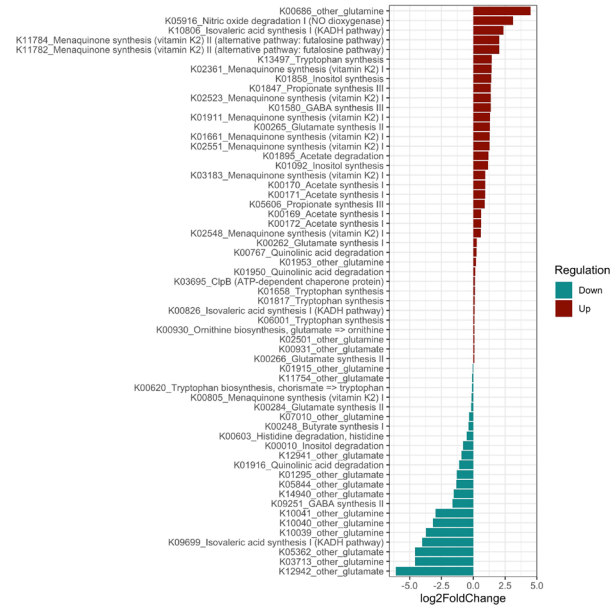
Figure 1

Figure 2

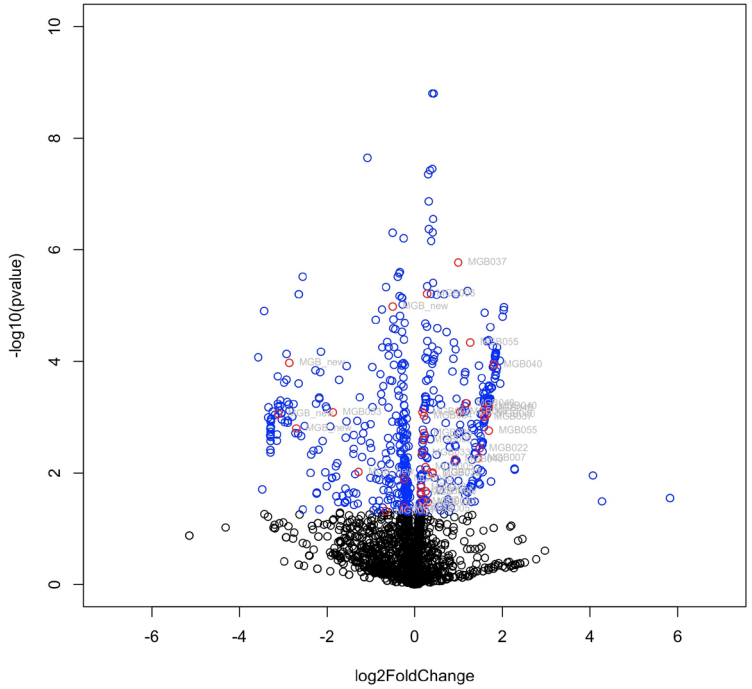
A



B



C



D

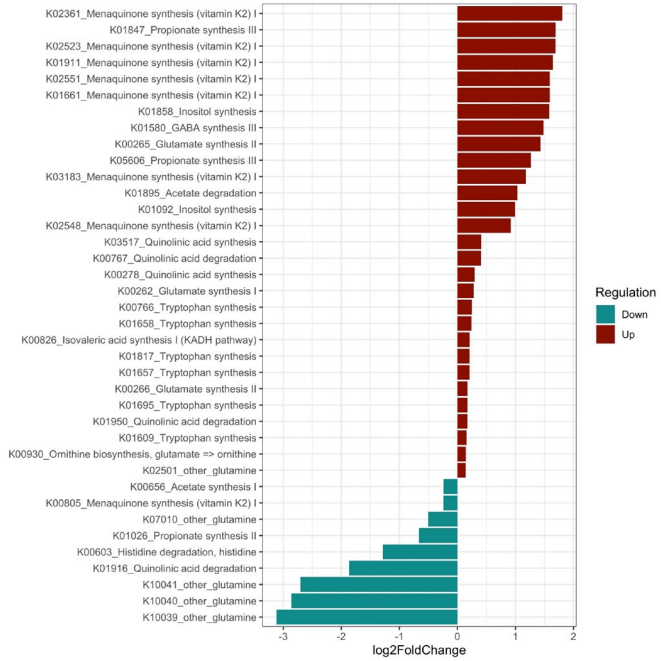


Figure 3

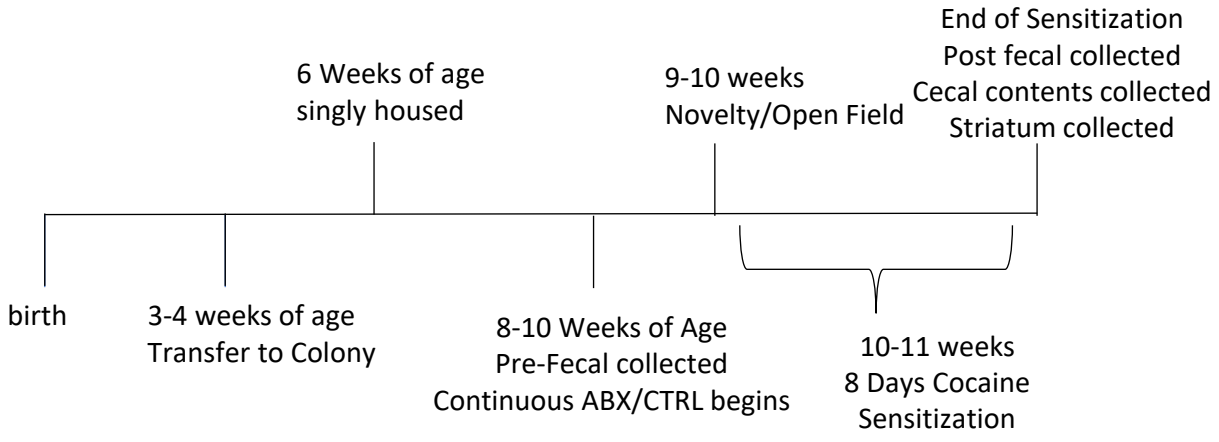

End of Sensitization
Post fecal collected
Cecal contents collected
Striatum collected
6 Weeks of age
singly housed
9-10 weeks
Novelty/Open Field
birth
3-4 weeks of age
Transfer to Colony
8-10 Weeks of Age
Pre-Fecal collected
Continuous ABX/CTRL begins
10-11 weeks
8 Days Cocaine
Sensitization

Figure 4

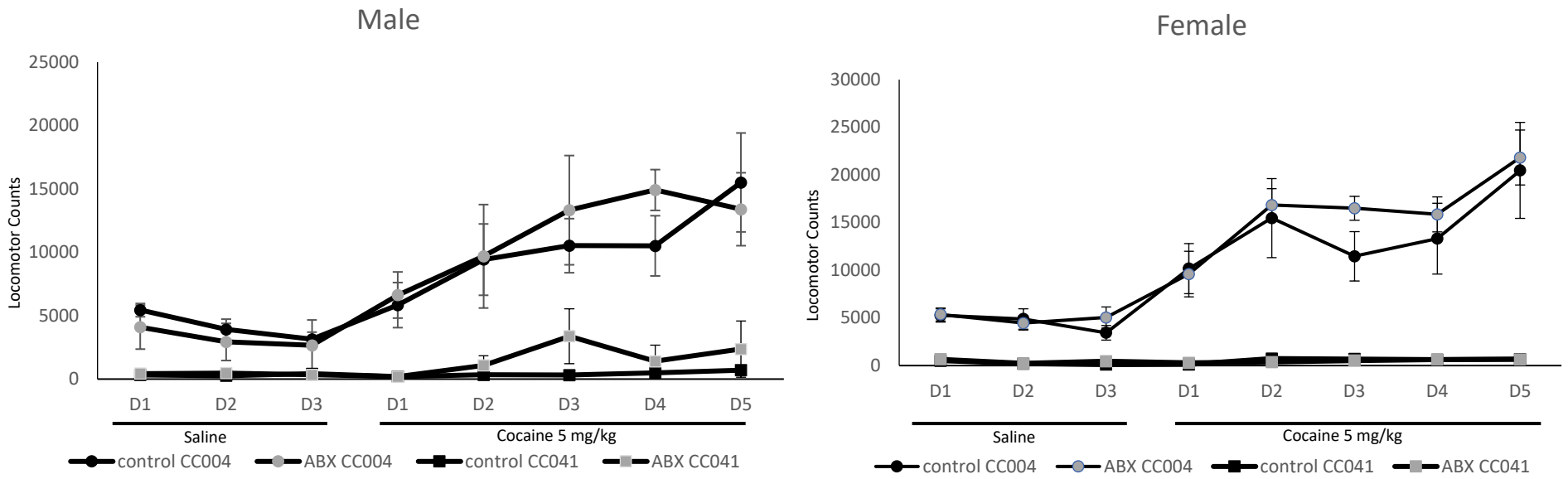
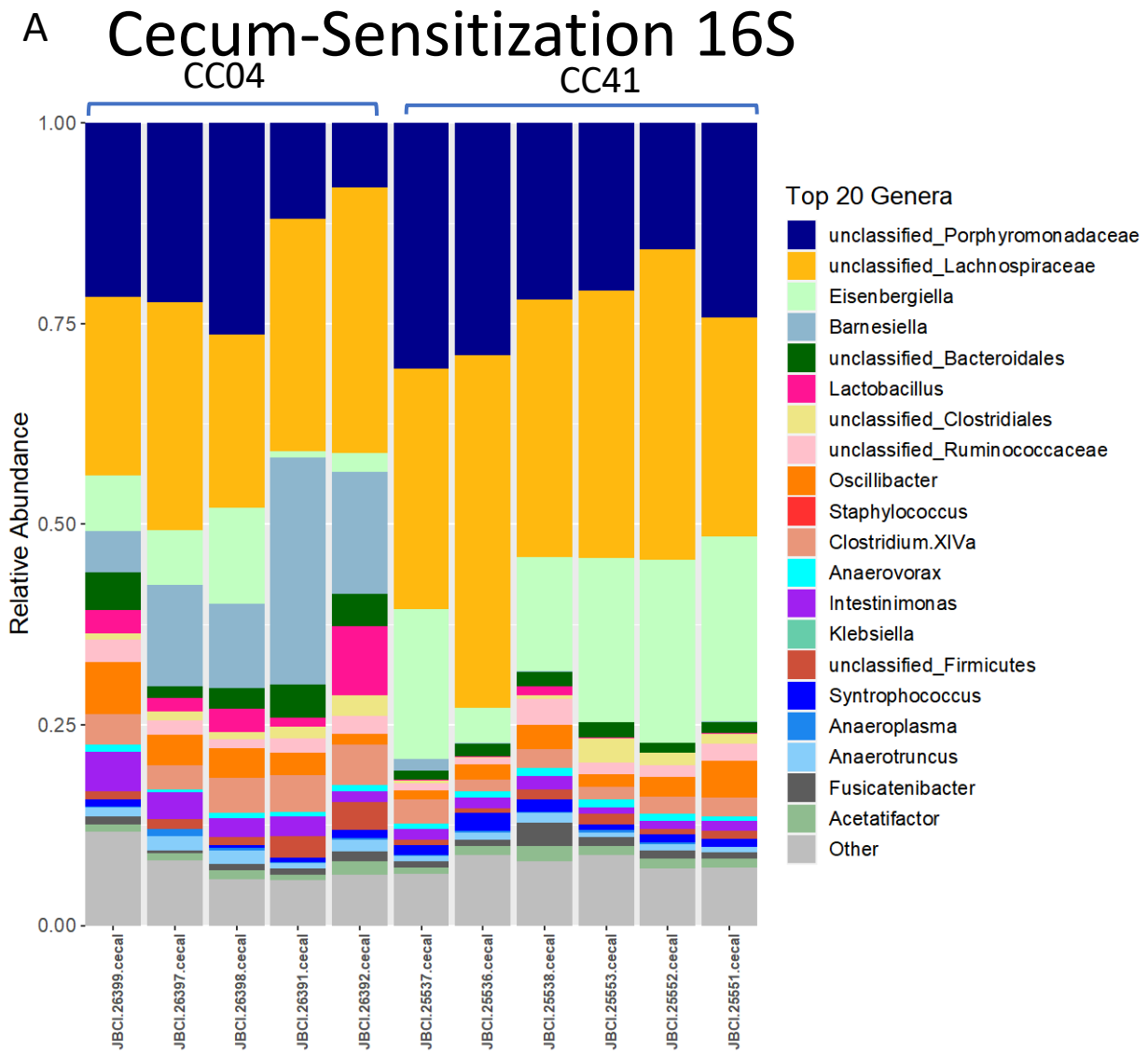

Male
Locomotor Counts
25000
20000
15000
10000
5000
0
D1
D2
D3
D1
D2
D3
D4
D5
Saline
Cocaine 5 mg/kg
control CC004
ABX CC004
control CC041
ABX CC041
Female
Locomotor Counts
30000
25000
20000
15000
10000
5000
0
D1
D2
D3
D1
D2
D3
D4
D5
Saline
Cocaine 5 mg/kg
control CC004
ABX CC004
control CC041
ABX CC041

Figure 5

A


Cecum-Sensitization 16S
CC04
CC41
Relative Abundance
1.00
0.75
0.50
0.25
0.00
JBCI.26399.cecal
JBCI.26397.cecal
JBCI.26398.cecal
JBCI.26391.cecal
JBCI.26392.cecal
JBCI.25537.cecal
JBCI.25536.cecal
JBCI.25538.cecal
JBCI.25553.cecal
JBCI.25552.cecal
JBCI.25551.cecal
Top 20 Genera
unclassified_Porphyromonadaceae
unclassified_Lachnospiraceae
Eisenbergiella
Barnesiella
unclassified_Bacteroidales
Lactobacillus
unclassified_Clostridiales
unclassified_Ruminococcaceae
Oscillibacter
Staphylococcus
Clostridium.XIVa
Anaerovorax
Intestinimonas
Klebsiella
unclassified_Firmicutes
Syntrophococcus
Anaeroplasma
Anaerotruncus
Fusicatenibacter
Acetatifactor
Other

B

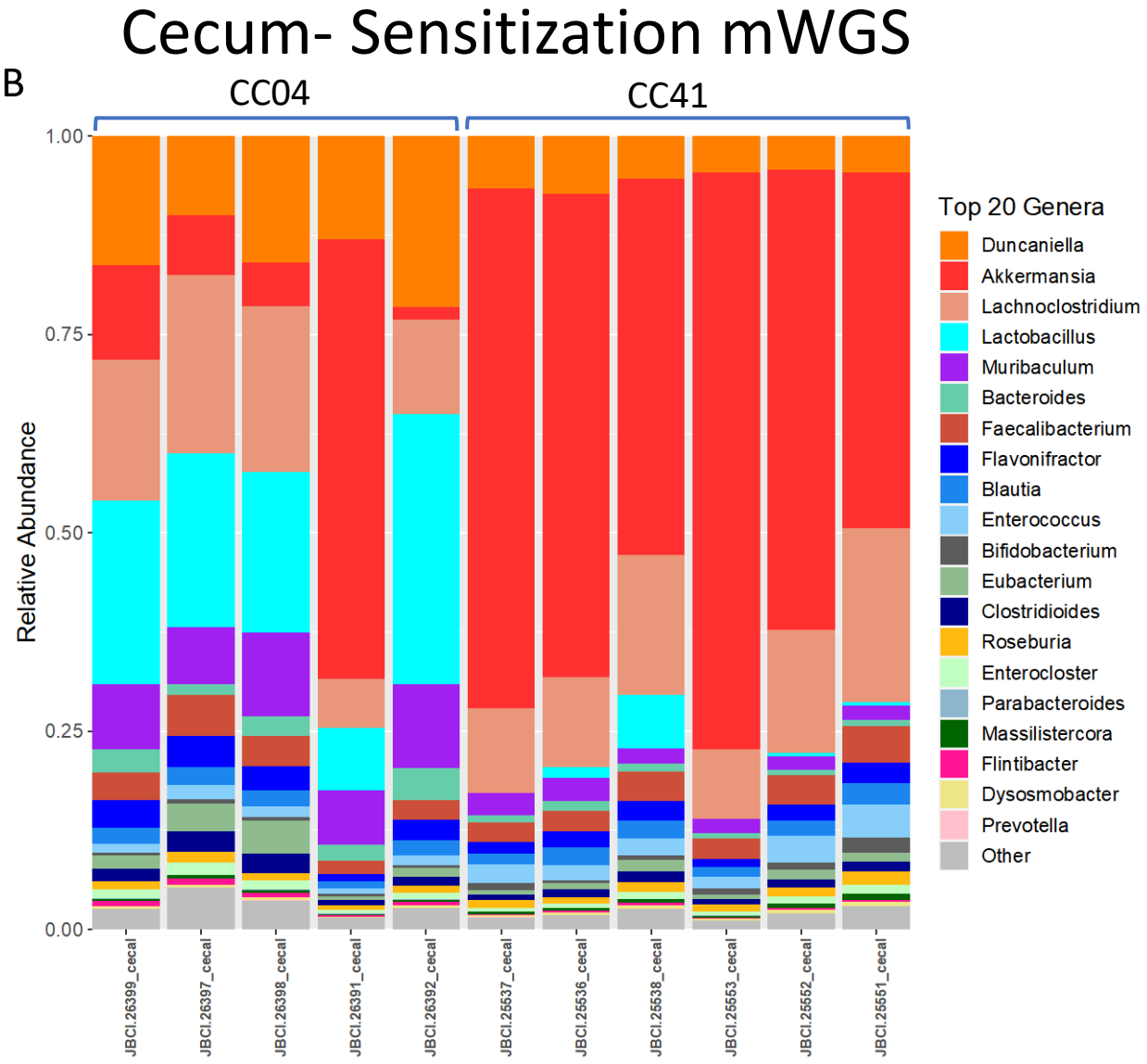
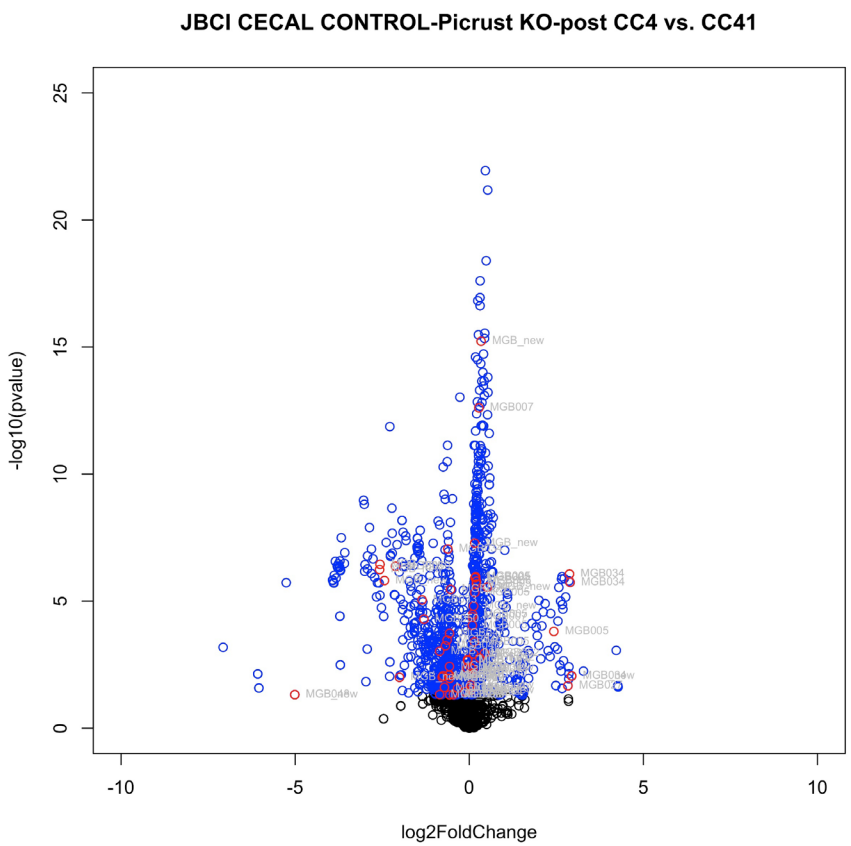

Cecum- Sensitization mWGS
CC04
CC41
Relative Abundance
1.00
0.75
0.50
0.25
0.00
JBCI.26399_cecal
JBCI.26397_cecal
JBCI.26398_cecal
JBCI.26391_cecal
JBCI.26392_cecal
JBCI.25537_cecal
JBCI.25536_cecal
JBCI.25538_cecal
JBCI.25553_cecal
JBCI.25552_cecal
JBCI.25551_cecal
Top 20 Genera
Duncaniella
Akkermansia
Lachnoclostridium
Lactobacillus
Muribaculum
Bacteroides
Faecalibacterium
Flavonifractor
Blautia
Enterococcus
Bifidobacterium
Eubacterium
Clostridioides
Roseburia
Enterocloster
Parabacteroides
Massilistercora
Flintibacter
Dysosmobacter
Prevotella
Other

Figure 6

A


JBCI CECAL CONTROL-Picrust KO-post CC4 vs. CC41
-log10(pvalue)
log2FoldChange
MGB_new
MGB007
MGB034
MGB005
MGB048w
MGB084w

B

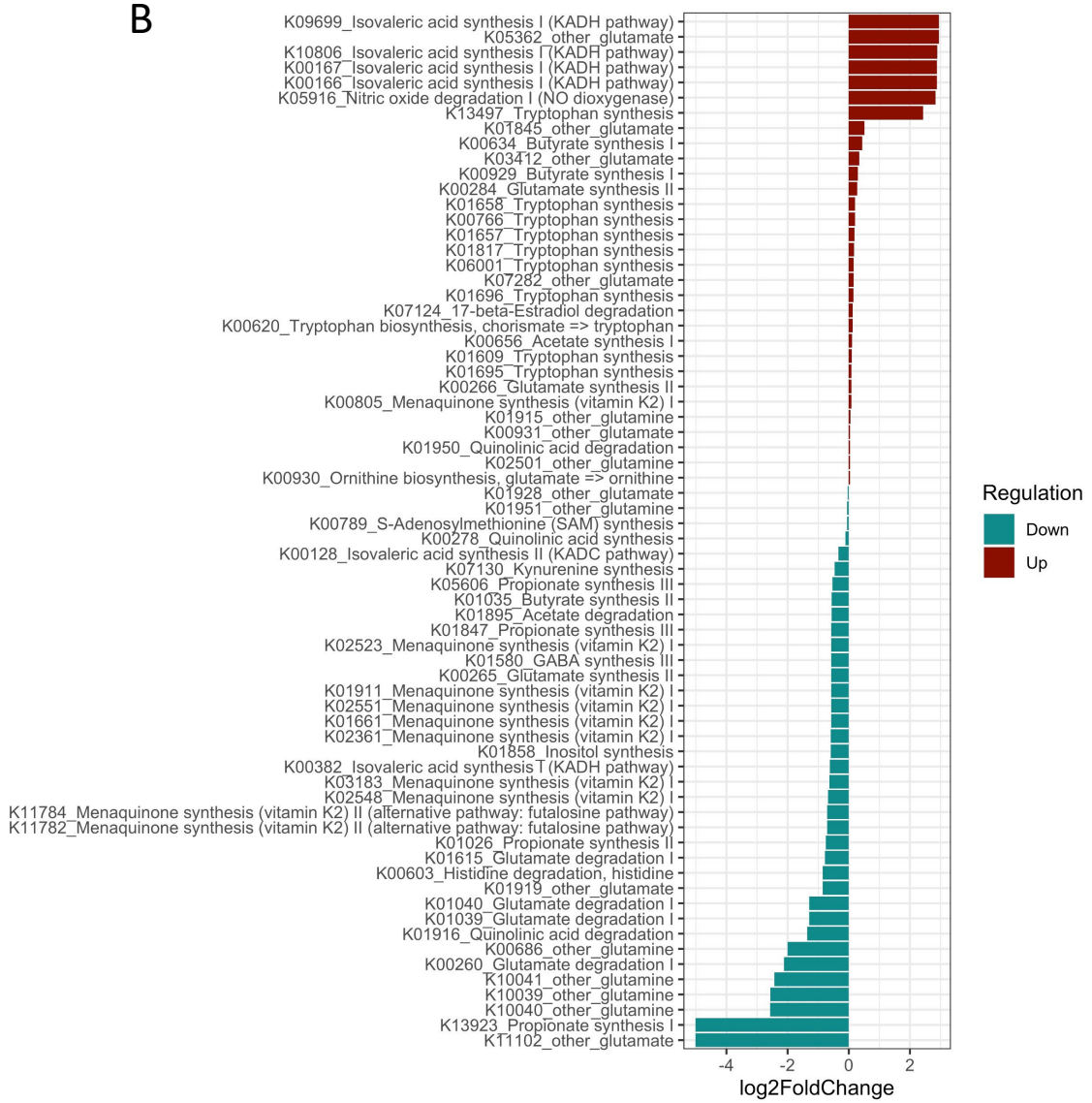
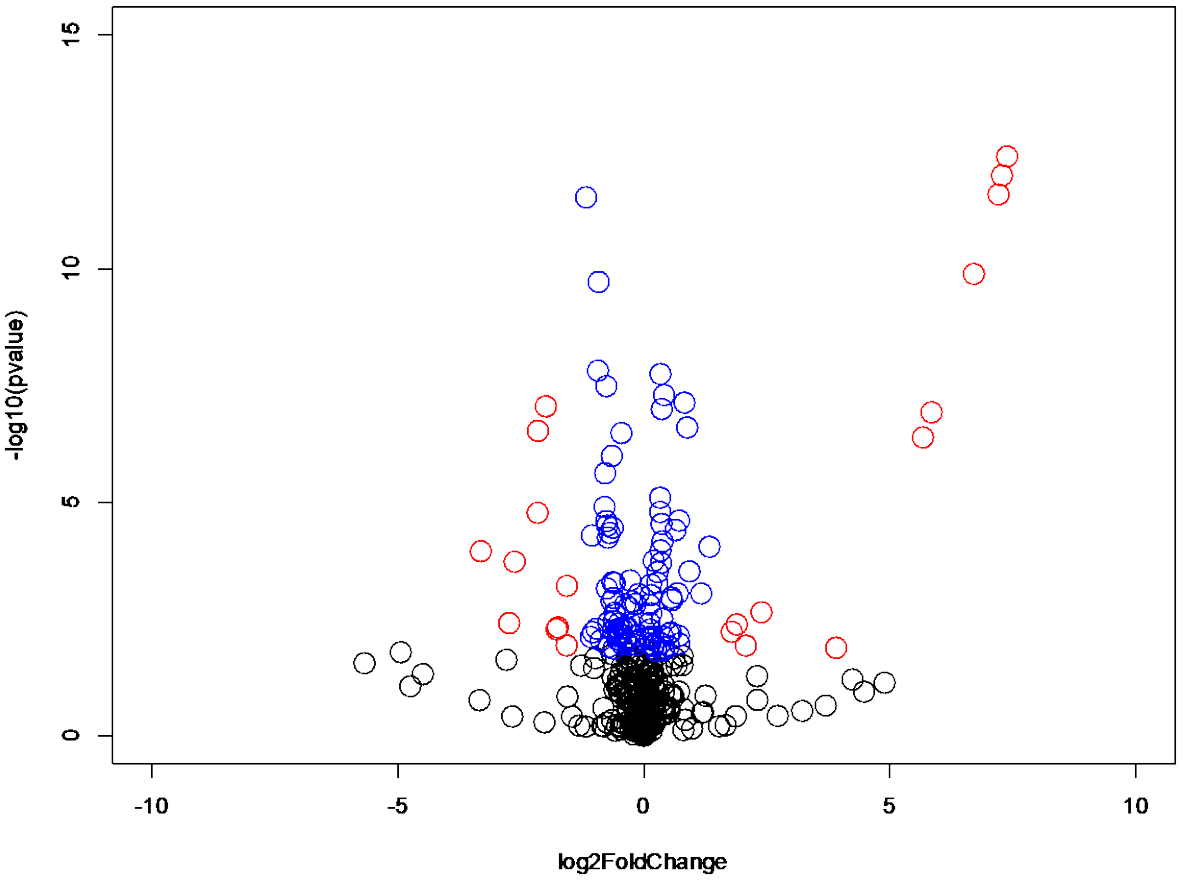

K09699_Isovaleric acid synthesis I (KADH pathway)
K05362_other_glutamate
K10806_Isovaleric acid synthesis I (KADH pathway)
K00167_Isovaleric acid synthesis I (KADH pathway)
K00166_Isovaleric acid synthesis I (KADH pathway)
K05916_Nitric oxide degradation I (NO dioxygenase)
K13497_Tryptophan synthesis
K01845_other_glutamate
K00634_Butyrate synthesis I
K03412_other_glutamate
K00929_Butyrate synthesis I
K00284_Glutamate synthesis II
K01658_Tryptophan synthesis
K00766_Tryptophan synthesis
K01657_Tryptophan synthesis
K01817_Tryptophan synthesis
K06001_Tryptophan synthesis
K07282_other_glutamate
K01696_Tryptophan synthesis
K07124_17-beta-Estradiol degradation
K00620_Tryptophan biosynthesis, chorismate => tryptophan
K00656_Acetate synthesis I
K01609_Tryptophan synthesis
K01695_Tryptophan synthesis
K00266_Glutamate synthesis II
K00805_Menaquinone synthesis (vitamin K2) I
K01915_other_glutamine
K00931_other_glutamate
K01950_Quinolinic acid degradation
K02501_other_glutamine
K00930_Ornithine biosynthesis, glutamate => ornithine
K01928_other_glutamate
K01951_other_glutamine
K00789_S-Adenosylmethionine (SAM) synthesis
K00278_Quinolinic acid synthesis
K00128_Isovaleric acid synthesis II (KADC pathway)
K07130_Kynurenine synthesis
K05606_Propionate synthesis III
K01035_Butyrate synthesis II
K01895_Acetate degradation
K01847_Propionate synthesis III
K02523_Menaquinone synthesis (vitamin K2) I
K01580_GABA synthesis III
K00265_Glutamate synthesis II
K01911_Menaquinone synthesis (vitamin K2) I
K02551_Menaquinone synthesis (vitamin K2) I
K01661_Menaquinone synthesis (vitamin K2) I
K02361_Menaquinone synthesis (vitamin K2) I
K01858_Inositol synthesis
K00382_Isovaleric acid synthesis I (KADH pathway)
K03183_Menaquinone synthesis (vitamin K2) I
K02548_Menaquinone synthesis (vitamin K2) I
K11784_Menaquinone synthesis (vitamin K2) II (alternative pathway: futalosine pathway)
K11782_Menaquinone synthesis (vitamin K2) II (alternative pathway: futalosine pathway)
K01026_Propionate synthesis II
K01615_Glutamate degradation I
K00603_Histidine degradation, histidine
K01919_other_glutamate
K01040_Glutamate degradation I
K01039_Glutamate degradation I
K01916_Quinolinic acid degradation
K00686_other_glutamine
K00260_Glutamate degradation I
K10041_other_glutamine
K10039_other_glutamine
K10040_other_glutamine
K13923_Propionate synthesis I
K11102_other_glutamate
log2FoldChange
Regulation
Down
Up

Figure 7

A


-log10(pvalue)
15
10
5
0
-10
-5
0
5
10
log2FoldChange

B

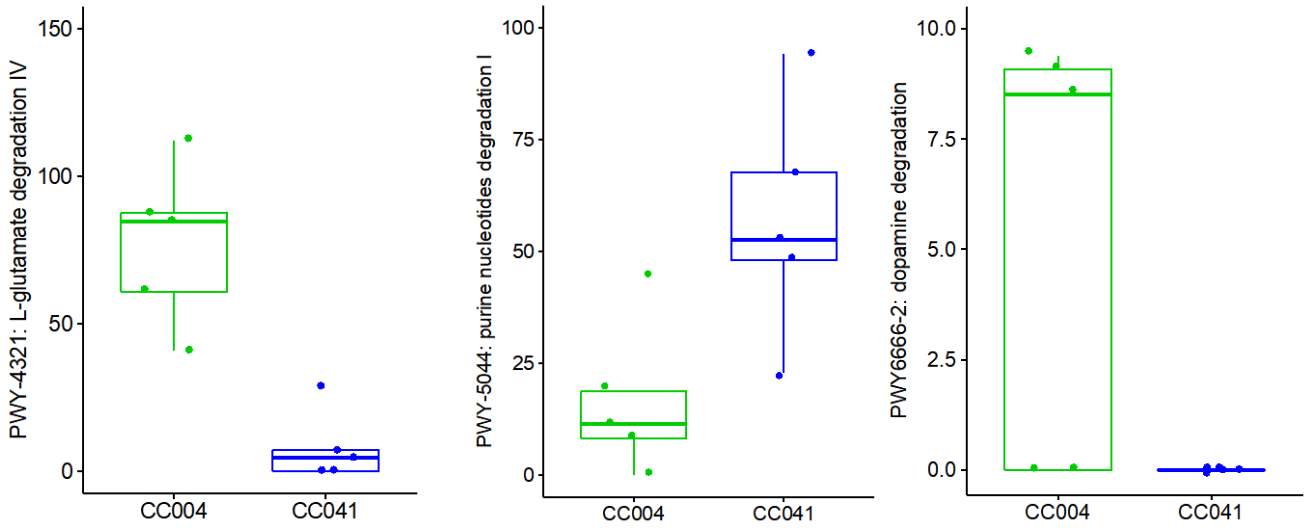

PWY-4321: L-glutamate degradation IV
150
100
50
0
CC004
CC041
PWY-5044: purine nucleotides degradation I
100
75
50
25
0
CC004
CC041
PWY6666-2: dopamine degradation
10.0
7.5
5.0
2.5
0.0
CC004
CC041

Figure S1

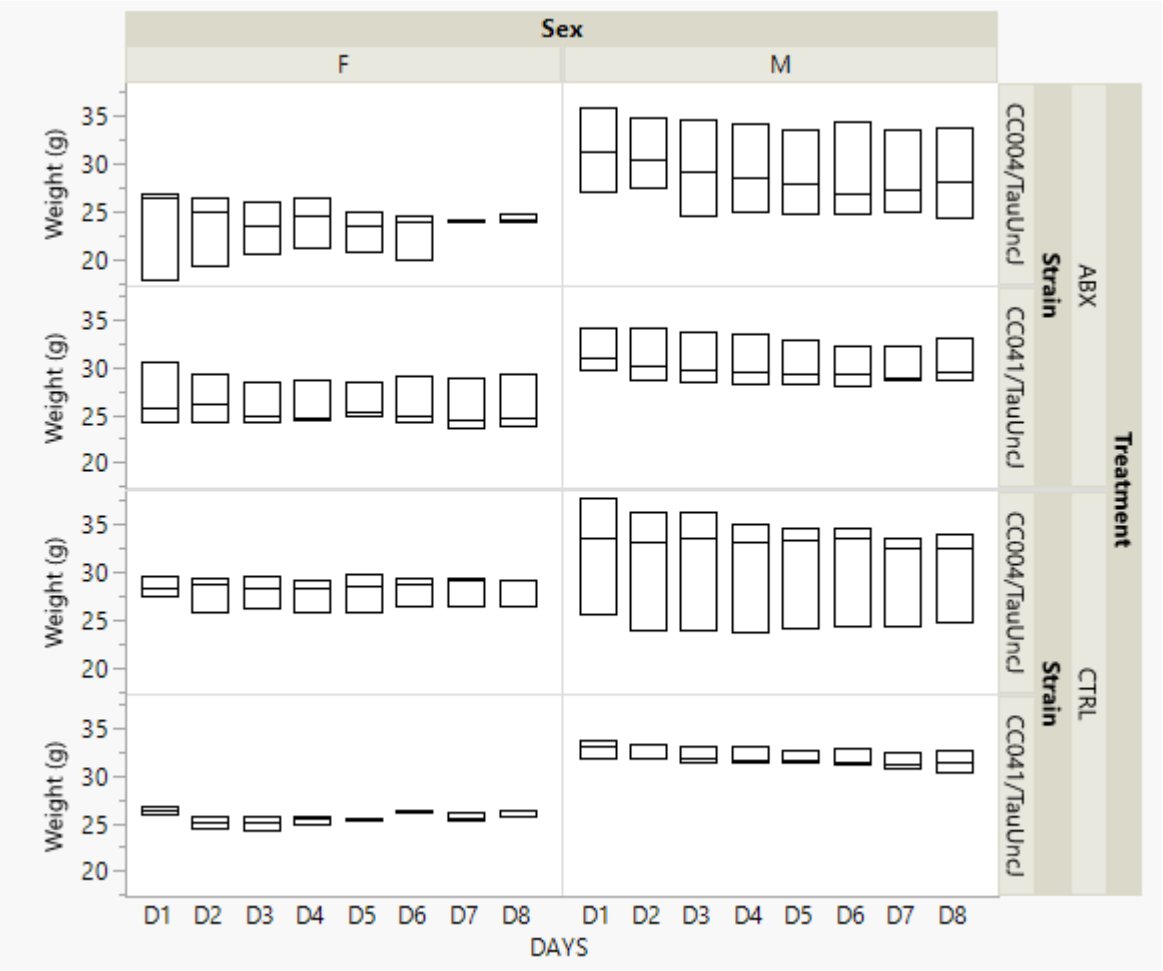

Sex
F
M
Weight (g)
35
30
25
20
D1
D2
D3
D4
D5
D6
D7
D8
DAYS
CC004/TauUncJ
CC041/TauUncJ
Strain
ABX
CTRL
Treatment

Figure S2

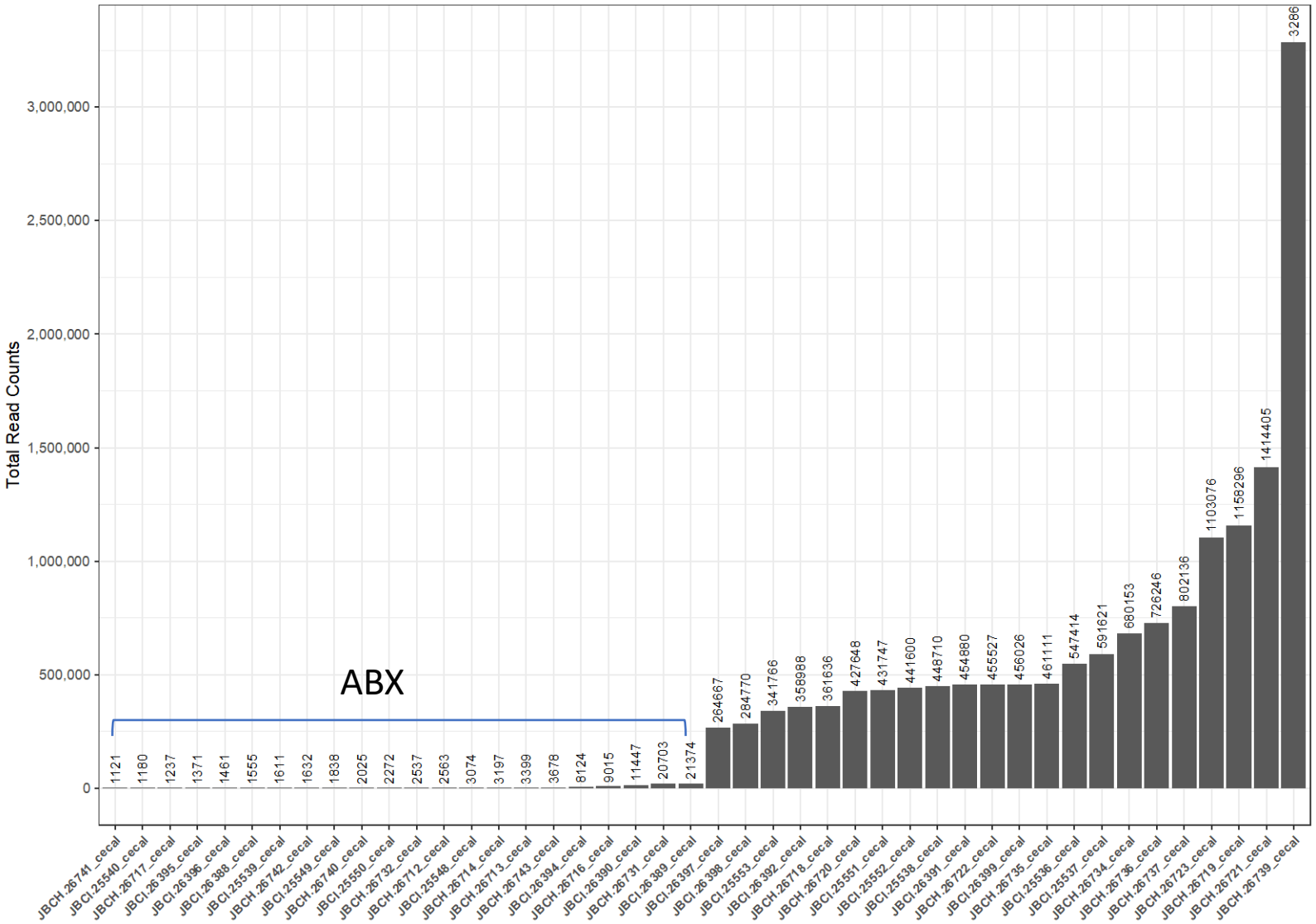
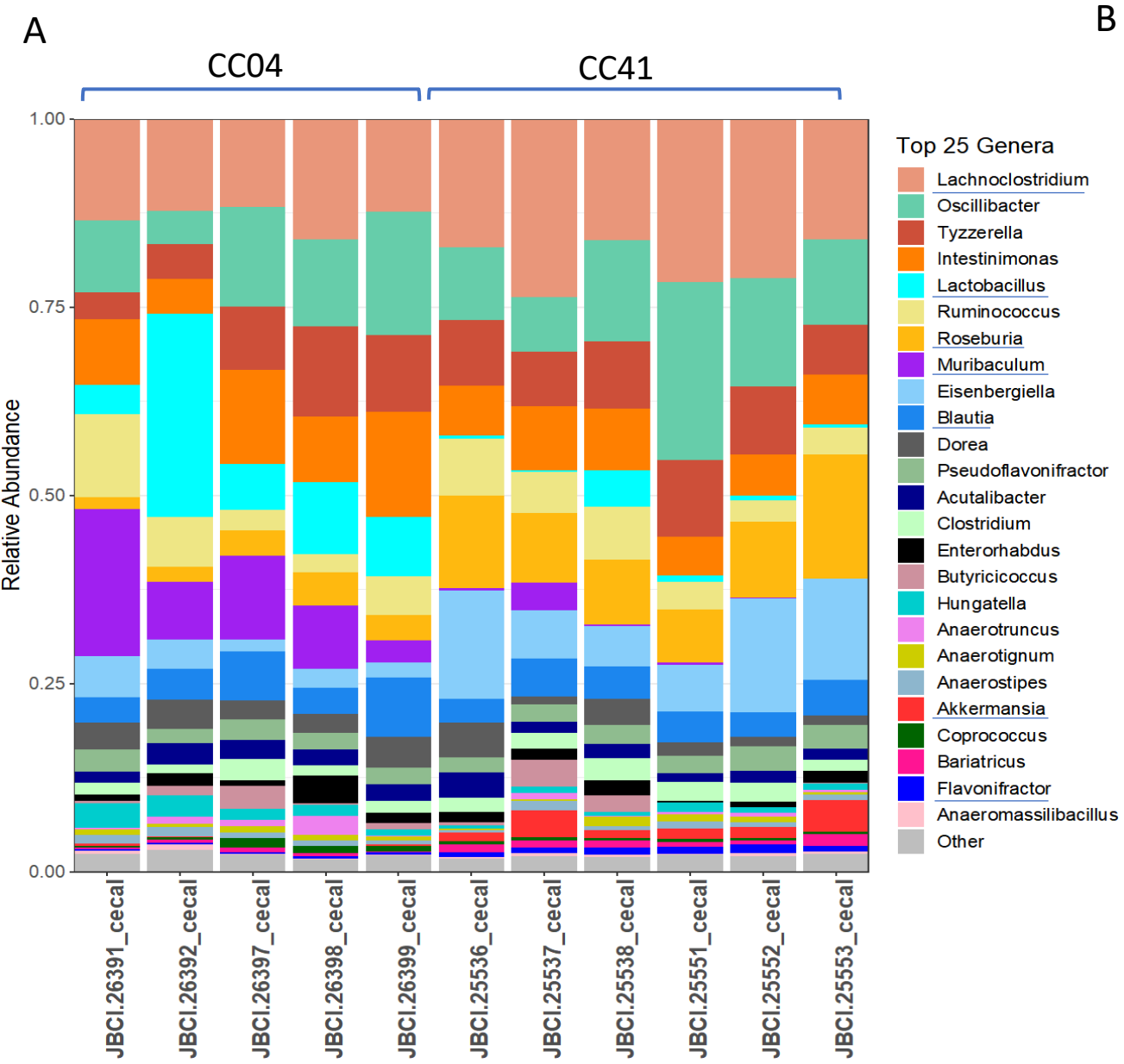
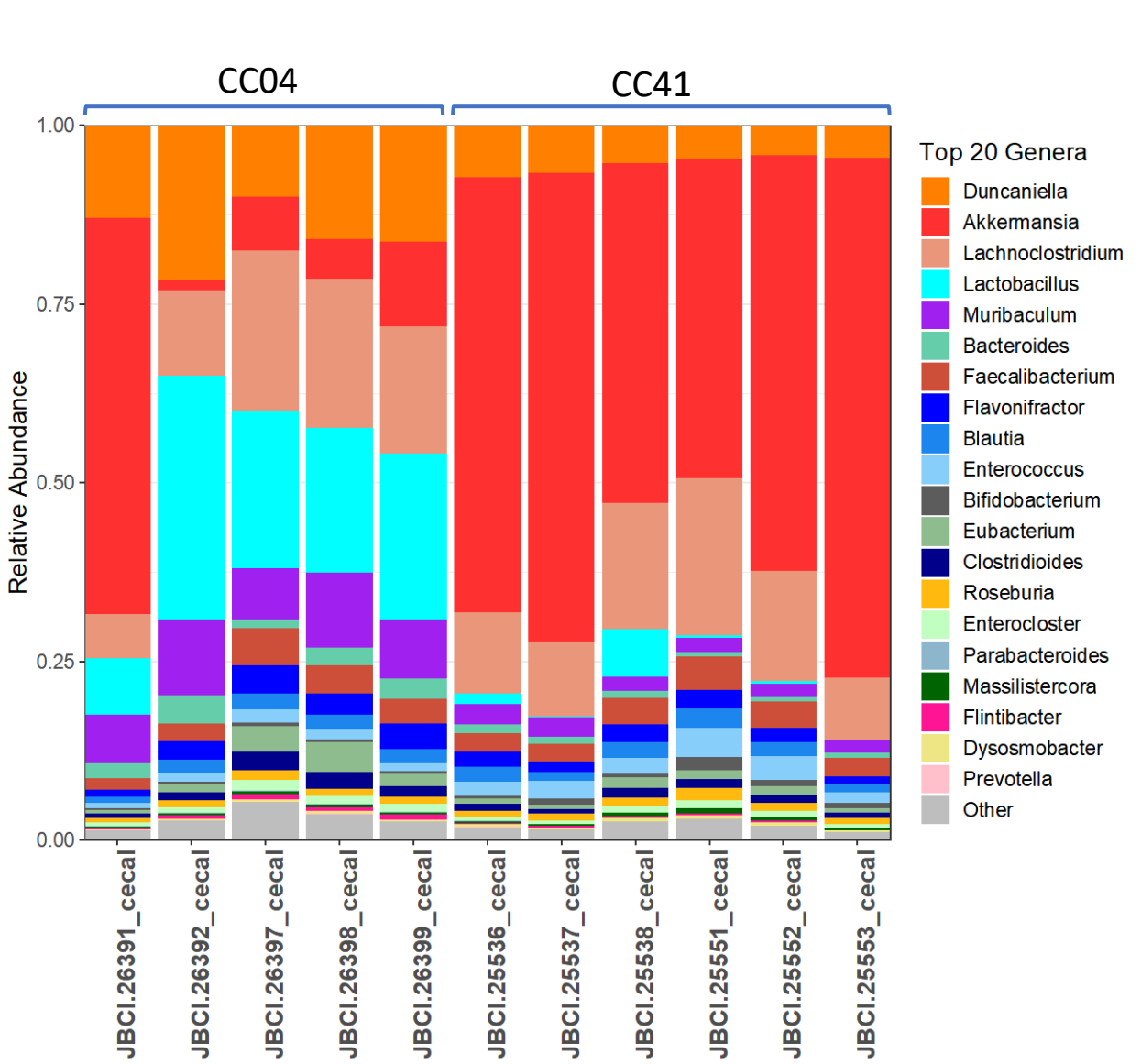

Total Read Counts
3,000,000
2,500,000
2,000,000
1,500,000
1,000,000
500,000
0
ABX
1121
1180
1237
1371
1461
1555
1611
1632
1838
2025
2272
2537
2563
3074
3197
3399
3678
8124
9015
11447
20703
21374
264667
284770
341766
358988
361636
427648
431747
441600
448710
454880
455527
456026
461111
547414
591621
680153
726246
802136
1103076
1158296
1414405
3286
JBCI.26741_cecal
JBCI.25540_cecal
JBCH.26717_cecal
JBCI.26395_cecal
JBCI.26396_cecal
JBCI.26388_cecal
JBCI.25539_cecal
JBCH.26742_cecal
JBCI.25549_cecal
JBCH.26740_cecal
JBCI.25550_cecal
JBCH.26732_cecal
JBCH.26712_cecal
JBCI.25548_cecal
JBCH.26714_cecal
JBCH.26713_cecal
JBCH.26743_cecal
JBCI.26394_cecal
JBCH.26716_cecal
JBCI.26390_cecal
JBCH.26731_cecal
JBCI.26389_cecal
JBCI.26397_cecal
JBCI.26398_cecal
JBCI.25553_cecal
JBCI.26392_cecal
JBCH.26718_cecal
JBCH.26720_cecal
JBCI.25551_cecal
JBCI.25552_cecal
JBCI.25538_cecal
JBCI.26391_cecal
JBCH.26722_cecal
JBCI.26399_cecal
JBCH.26735_cecal
JBCI.25536_cecal
JBCI.25537_cecal
JBCH.26734_cecal
JBCH.26736_cecal
JBCH.26737_cecal
JBCH.26723_cecal
JBCH.26719_cecal
JBCH.26721_cecal
JBCH.26739_cecal

Figure S3


A
CC04
CC41
Relative Abundance
1.00
0.75
0.50
0.25
0.00
JBCI.26391_cecal
JBCI.26392_cecal
JBCI.26397_cecal
JBCI.26398_cecal
JBCI.26399_cecal
JBCI.25536_cecal
JBCI.25537_cecal
JBCI.25538_cecal
JBCI.25551_cecal
JBCI.25552_cecal
JBCI.25553_cecal
Top 25 Genera
Lachnoclostridium
Oscillibacter
Tyzzerella
Intestinimonas
Lactobacillus
Ruminococcus
Roseburia
Muribaculum
Eisenbergiella
Blautia
Dorea
Pseudoflavonifractor
Acutalibacter
Clostridium
Enterorhabdus
Butyricicoccus
Hungatella
Anaerotruncus
Anaerotignum
Anaerostipes
Akkermansia
Coprococcus
Bariatricus
Flavonifractor
Anaeromassilibacillus
Other


B
CC04
CC41
Relative Abundance
1.00
0.75
0.50
0.25
0.00
JBCI.26391_cecal
JBCI.26392_cecal
JBCI.26397_cecal
JBCI.26398_cecal
JBCI.26399_cecal
JBCI.25536_cecal
JBCI.25537_cecal
JBCI.25538_cecal
JBCI.25551_cecal
JBCI.25552_cecal
JBCI.25553_cecal
Top 20 Genera
Duncaniella
Akkermansia
Lachnoclostridium
Lactobacillus
Muribaculum
Bacteroides
Faecalibacterium
Flavonifractor
Blautia
Enterococcus
Bifidobacterium
Eubacterium
Clostridioides
Roseburia
Enterocloster
Parabacteroides
Massilistercora
Flintibacter
Dysosmobacter
Prevotella
Other