Title

Sex-dependent associations between addiction-related behaviors and the microbiome in outbred rats

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Sex, Drugs, and the Microbiome: Goal/Sign-Tracking Phenotype Reveals Associations between Behavior and Microbiome in a Sex-Dependent Manner in the Rat

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Abstract

Introduction: Multiple factors contribute to the etiology of addiction, including genetics, sex, and a number of addiction-related behavioral traits. For example, individuals that are predisposed to assign incentive salience to food stimuli ("sign-trackers", ST), are not only more impulsive compared to those that do not ("goal-trackers", GT), but are also more sensitive to drugs and drug stimuli as well. Recent studies have implicated the gut microbiota as a key regulator of brain and behavior, and have shown that many microbiota-associated changes occur in a sex-dependent manner. However, few studies have examined how the microbiome might influence addiction-related behaviors. To this end, we sought to determine if gut microbiome composition was correlated with addiction-related behaviors.

Methods: Outbred male (N=101) and female (N=101) heterogeneous stock rats underwent a series of behavioral tests measuring impulsivity, attention, reward-learning, incentive salience, and locomotor response. Cecal microbiome composition was estimated using 16S rRNA. Behavior and microbiome were characterized and correlated with behavioral phenotypes. Robust sex differences were observed in both behavior and microbiome; further analyses were conducted within sex using the pre-established goal/sign-tracking (GT/ST) phenotype and partial least squares differential analysis (PLS-DA) clustered behavioral phenotype.

Results: Microbial alpha diversity was significantly decreased in female STs. On the other hand, a measure of impulsivity had many significant correlations to microbiome in both males and females. Several measures of impulsivity were correlated with the genus *Barnesiella* in females. In the female STs, attention, as measured by omissions in the reaction time test revealed notable microbiome correlations. In both males and females, many measures were correlated with bacterial family Ruminococcaceae and Lachnospiraceae.

Conclusions: These data demonstrate correlations between several addiction-related behaviors and the microbiome specific to sex.

1. Introduction

Addiction is a complex disorder; many factors modulate addiction severity and treatment efficacy. Extensive research has identified socioeconomic status (Heilig et al., 2016), childhood trauma (Enoch et al., 2010; Schwandt et al., 2017), genetics (Palmer and de Wit, 2012), sex (Becker and Hu, 2008), and psychological comorbidities as factors that contribute to addiction risk and severity (Grant et al., 2015). Due to the complex etiology of addiction, treatment is a trial and error process, frequently requiring a combination of therapies (Heilig et al., 2011). More research is necessary to investigate how genetic and environmental factors contribute to addiction. Additionally, new factors such as the effect of diet, microbiome, and social interventions require greater attention. In particular, manipulation of the gut microbiome offers an intriguing target for new addiction treatments (Temko et al., 2017).

The gut microbiota consists of a myriad of bacteria, archaea, fungi, and viruses colonizing the host gastrointestinal tract and influencing many host systems, such as metabolism (Barton et al., 2017; Ryan et al., 2017), immune function (Kau et al., 2011; Kundu et al., 2017), hypothalamic-pituitary-adrenal axis response, and brain (Collins et al., 2013; Hsiao et al., 2013; Mayer et al., 2015; Sherwin et al., 2017). The gut microbiota is defined as the community of microbes residing in the intestines; the microbiome is the genomic DNA from all microbes in that community. In the context of affective disorders, both animal and human studies have strongly linked the composition of the gut microbiota to the behavioral aspects of these disorders (Bercik et al., 2010; Bravo et al., 2011; Collins et al., 2013; Kelly et al., 2016; Zheng et al., 2016). Furthermore, microbiota has been linked to psychiatric disorders that involve an array of behavioral abnormalities, such as autism spectrum disorder (Golubeva et al., 2017; Hsiao et al., 2013; Mayer et al., 2014). Previous research has indicated a link between gut microbiome, addiction, and drugs of abuse (Kiraly et al., 2016; Leclercq et al., 2014b; Ning et al., 2017; Peterson et al., 2017; Scheperjans et al., 2015; Yan et al., 2011).

Drugs of abuse activate ‘the reward pathway’, which includes cortical innervations in the ventral tegmental area (VTA), striatum, and prefrontal cortex (PFC). Behavioral measures that have been developed in rodents to explore this pathway, include: locomotor response to novelty (Flagel et al., 2011), measures of impulsivity (Reynolds et al., 2006), attention (Gancarz et al., 2012), and reward-stimulus learning (Flagel and Robinson, 2017). In the case of reward-stimulus learning, the unconditioned stimuli (USs) and the conditioned stimuli (CSs) can activate this reward pathway (Schultz et al., 1997). Individual differences in the activation of this pathway promote differential behavioral responsivity to CSs (Flagel et al., 2011); "sign-tracking" rats (STs) approach CSs more than their "goal-tracking" counterparts (GTs)(Flagel et al., 2007; Meyer et al., 2012a). Additionally, STs and GTs are

differentially sensitive to the motivational properties of several abused drugs (Saunders and Robinson, 2010; Versaggi et al., 2016; Yager et al., 2015; Yager and Robinson, 2015). Furthermore, STs are more impulsive as measured by test of impulsive action and choice (King et al., 2016; Lovic et al., 2011; Meyer and Tripi, 2018; Tomie et al., 2008). Interestingly, sign-tracking behavior is more frequent in females than male rats (King et al., 2016; Pitchers et al., 2015). Impulsivity has also been associated with increases in addiction-related behavior, as well as attention deficit disorder in both rodents (Gancarz et al., 2011; King et al., 2016) and humans (Sanchez-Roige et al., 2018).

The relationship between sex hormones, microbiome and behavior is gaining attention (Amato et al., 2014; Chaban et al., 2014; Jasarevic et al., 2016; Schnorr et al., 2014). During development factors like genetics and hormones, contribute to sexual dimorphism and associated to sex-differences in microbiome (Jasarevic et al., 2016). Clinical and pre-clinical research has shown differences in microbiota composition associated with altered metabolism of essential vitamins and nutrients from the diet, with increased metabolic function seen in females compared to males (Amato et al., 2014; Bolnick et al., 2014; Zhernakova et al., 2016). Worldwide, illicit drug use is significantly lower in females compared to males (World Health Organization, 2012). However, females self-administer drugs more readily than males and are more vulnerable to addiction to a variety of licit and illicit drugs (Becker and Hu, 2008; Yang et al., 2017).

This study, to our knowledge, is the first to investigate sex-specific relationships between addiction-related behavioral measures and the microbiome in a large dataset. The pre-established GT/ST phenotype was characterized for this study and correlations between behavioral measures and microbiome are described. These results are presented alongside a clustered behavioral phenotype constrained by goal/sign-tracker phenotype. Differences in behavioral measures and microbiome was characterized for each phenotype, within sex.

2. Methods

2.1 Animals

Male and female heterogenous stock (HS) rats were bred at Medical College of Wisconsin and then shipped to the University at Buffalo for behavioral testing. This National Institutes of Health (NIH)-derived outbred rat colony shows broad phenotypic and genotypic variation (Parker et al., 2014), making it an ideal choice for the study of individuals differences. Rats (N=202) were housed in pairs in plastic cages (42.5 cm × 22.5 cm × 19.25 cm); males and females housed in the same room in alternating cages in testing order. In the event of odd numbers of rats, rats were housed individually. Animals were kept in reversed 12-h light/dark cycle and housed in controlled temperature and humidity conditions. Lights were on in the colony room from 19:00 pm to 07:00 hours. Behavioral testing occurred 6 days/week between of 08:30 and 12:30 hours during the dark phase of the light cycle. Food (#8604, Harlan Inc., Indianapolis, IN) was continuously available. Animals were treated in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University at Buffalo, The State University of New York.

2.2 Behavioral tests

Behavioral testing was carried out on rats beginning at 63 days of age (see Table 1). All behavioral tests were conducted in the dark phase of the light cycle. Epochs for behavioral tests consisted of consecutive 3 minute intervals across a test session.

2.2.1 Locomotor Response to Novelty (Loco)

To assess locomotor response to novelty, rats were placed in a 24x45cm clean plastic standard laboratory cage. A Hamilton Kinder motor monitor frame contained infrared photo detectors which measured locomotor activity by beam breaks. Rats were placed into the test cages for one hour. Only the first 18 minutes of the 1-hour test session were used for analysis. Each rat was tested only once. Measures used for this test included total locomotor activity (Loco.Activity), epoch with the greatest activity (Loco.MaxAct), total distance travelled (Loco.Distance), epoch with the greatest distance travelled (Loco.MaxDist), time in center (Loco.Center), total number of rears (Loco.Rear), epoch with the greatest number of rears (Loco.MaxRear).

2.2.2 Light Reinforcement (LR)

In-house constructed operant chambers were used for testing (see Supp. Methods Fig 2). The visual stimulus (VS) reinforcer used in the experiment was the onset of the light located in the middle of the

back wall of the test chamber. Onset of the VS reinforcer produced an illuminance of 68 lx, as measured from the center of the test chamber. The VS reinforcer was illuminated for 5 s each time it was presented. Each test chamber was housed in a Coleman Cooler (Model # 3000000187), which blocked external audiovisual sources of stimulation. The pre-exposure phase consisted of six 18 min sessions. Light reinforcement testing took place immediately after pre-exposure testing with one day off in-between. The light reinforcement phase consisted of six 18 min sessions. During light reinforcement testing, rats were placed in dark experimental chambers and snout pokes into the aperture designated as 'active' resulted in 5 s illumination according to a variable interval 1-minute (VI1) schedule of reinforcement. Measures from light reinforcement testing included total number of light reinforcers (LR.Reinforcers), total number InActive responses during test (LR.InActive), total number of Active and InActive responses during test (LR.Total), epoch with greatest total responses (LR.TotMax) and epoch with greatest InActive responses (LR.InActMax).

2.2.3 Choice Reaction Time Task (RT)

Locally constructed experimental chambers were used for the choice reaction time task. The test panel had two water dispensers located on either side of a centrally located snout-poke hole (see Supp. Methods Fig 3). The water dispenser and stimulus lights were arranged so that they were level with the rat's eyes when the rat's snout interrupted an infrared beam in the center snout-poke hole. Rats were placed into the test cages for 18 minutes for each test session. Rats initiated trials by holding their snout in the center snout hole until the left stimulus light was turned on (hold time). Once the hold time criterion was reached and the imperative stimulus was presented, the rat had 3 seconds to respond by removing its snout and inserting it into the left feeder hole (reaction time), or the trial ended and the trial was counted as an omission. If the rat made a correct response, the rat received a water reinforcer (30 µl) and the trial ended. A false alarm was recorded when the rat pulled its snout out of the center hole to respond to the left water feeder hole prior to the onset of the imperative stimulus. Premature initiations are defined similar to a false alarm, except that the rat pulls out of the center snout poke hole before the imperative stimulus occurs and then puts its snout back into the center hole without going to the left water feeder hole. The final 3 test sessions were used for analysis. Measures for this test included total number of correct responses (RT.Corr), mean reaction time (RT.MeanRT), per opportunity (trial) premature initiations (RT.PerOPInit), per opportunity false alarms (RT.POFA), and total omissions (RT.Omissions).

2.2.4 Delay Discounting (DD)

Delay Discounting was measured using a sequential patch depletion procedure. This procedure mimics naturally occurring choice problems confronting animals while foraging in resource scarce environments (i.e., travel delays and patch depletion). Behavior was measured in in-house constructed operant chambers (see Supp. Methods Fig 2). In the laboratory patch depletion procedure rats drink water at both the left and center water feeders. Rats receive successively smaller amounts of water every 4 s by remaining at the same feeder. The amount of water is initially 150 µL and is then decreased by 20% after each delivery from the same feeder. The rats can reset the amount of water to the initial maximum of 150 µL by switching to the alternative water feeder. However, changing to a new patch results in a delay to activation of the new feeder (travel time delay). During the delay, water is not available at either feeder. A change in patch is indicated by a snout poke into the alternative non-active feeder (patch). The indifference point (IDPt) is defined as the amount of water available at the current feeder (or patch) when the rat chose to switch to the new patch. Test sessions last for 10 minutes or until the rats consumed a cumulative total 5 ml of water, which ever occurred first. The area under the curve (AUC) was calculated for successive session measures. Behavioral measures from this test included indifference point area maximum (DD.IDPMax) and under the curve (DD.IDPtAUC), patch change rate area under the curve (DD.PCRateAUC), and average reinforcer rate (DD.RFRate).

2.2.5 Pavlovian Conditioned Approach (PavCA) and Reinforcement (CRF)

To examine individual differences in the propensity to attribute incentive salience to reward cues, HS rats were first exposed to a Pavlovian conditioning paradigm wherein a cue (lever) is repeatedly paired with presentation of a reward (food). Before animals undergo the standard PavCA procedure, they receive ~25 banana-flavored food pellets (Envigo, #F0059) in their homecages for 2 days. Then they undergo one day of magazine training, during which they are placed into Med-associates conditioning chambers and food pellets are delivered on a VI 30 s (1-60 s) schedule. For the subsequent 5 PavCA conditioning days, rats receive 25 CS-->US conditioning trials, presented on a VI 90 s (30-150 s) schedule. During each trial, an 8-s presentation of an illuminated lever CS preceded the delivery of a food pellet. On the day following the final session of PavCA, we perform conditioned reinforcement (CRF) in which rats can nose poke for presentations of the lever CS. All variables are derived from lever presses and magazine entries, including latencies and probability. Measures for PavCA included index scores on day 4 and 5 [see (Meyer et al., 2012a) for a description of this index]; briefly, scores range from -1 to 1 with negative numbers indicating magazine directed responses (goal-tracking), and positive numbers indicating lever-CS directed responses (sign-tracking). CRF measures used were total number of lever presses (CRF.LeverPresses) and total number of active nose-poke port entries (CRF.ActivePort) and total number of CS lever presentations (CRF.Reinforcers).

2.2.6 Cocaine Cue Preference (CCP)

To examine the individual differences in approach to a cocaine-associate cue, HS rats were tested for their response to a cocaine paired tactile cue [see (Meyer et al., 2012b) for details]. Briefly, testing chambers were constructed with black acrylic walls (47.5 cm length x 15.5 cm width x 30 cm height) with black spray-painted textured floors that were either stainless steel rods (“grid”) or perforated steel (“hole”), or half grid and half hole. After one day of habituation to the chamber and one "pre-test" day to determine grid-hole preferences, rats were given four conditioning trials. Each trial was two days, one saline-paired day and one cocaine-paired day. On saline-paired days, rats were given an injection of saline (i.p.) and placed into a chamber with either a uniform grid or hole floor (whichever was their preferred floor). On the cocaine paired day, rats are given an injection of cocaine (10 mg/mL; Nat. Inst. Of Drug Abuse, Bethesda, MD) and placed in a chamber with the opposite uniform floor (i.e. non-preferred floor). On the last "post-test" day, rats were given a saline injection, and presented with both cocaine- and saline-paired floors. The time spent on each floor was analyzed to determine cocaine cue preference. The primary dependent measures are change in time spent on the cocaine paired floor from pre-test to post-test (CCP.PreTest.Time.CS - CCP.PostTest.Time.CS = CCP.dtCS). Secondary measures include cocaine change in locomotor activity on trails 1 and 4 (CCP.T1.Cocaine.Dist CCP.T4.Cocaine.Dist).

2.3 Cecal microbiome collection and sequencing

Cecum was collected and snap-frozen on dry ice. Protocols for 16S rRNA microbiome sequencing were used as previously described (Peterson et al., 2017). Briefly, cecal contents from frozen cecum (stored at -80ºC) were extracted under a sterile hood. The QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract bacterial DNA from cecal contents using the manufacturer’s handbook (Second Edition 2012). Samples were prepared for 16S sequencing using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), as described in the Illumina 16S library preparation workflow. 16S bacterial rRNA gene was amplified using primers targeting the V3-V4 hypervariable region (Forward: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; Reverse: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (Sigma Aldrich Ireland ltd., Wicklow, Ireland). The Illumina V3–V4 primers were selected for their high coverage (94.5% bacteria) while remaining in the amplicon size necessary for sequencing to sequence at 2 × 250 bp (Klindworth et al., 2013). 16S rRNA amplicons were sequenced on the Illumina MiSeq platform, multiplexed on 4 separate runs (~50 samples per run) (Teagasc, Moorepark, Ireland).

2.4 Microbiome Sequence Processing

All sequences in FASTQ files format were filtered using PRINSEQ. Sequences with length less than 150 nucleotides or with low quality at the 3' end were removed. Paired-end reads with a minimum overlap of 20 base-pairs were joined using FASTQ-join and analyzed with QIIME (Quantitative Insights Into Microbial Ecology, v1.9.1). Sequence quality was checked and chimeras removed, remaining sequences were clustered into Operational Taxonomic Units (OTUs ; 97% identity level) using USEARCH (Version 7.0-64bit). The average number of high-quality sequences generated per sample was 153,561 ± 84,269 SD. Taxonomy was assigned to OTUs using Silva version 123. Alpha diversity (Observed, Chao1, Shannon, Simpson) indices were calculated with QIIME.

2.5 Statistical Analysis

Data was analyzed in R (v3.3.3) and RStudio (v1.0.136). Plots were generated in R using ggplot2 package (v2.2.1). All testing was corrected for multiple comparisons using the qvalue R package (v2.6.0). For OTU correlations to behavior, q-value confidence interval was set to 0.15. For within-sex correlations, the q-value of 0.15 was accepted due to the exploratory nature of this study. In this dataset, q=0.15 indicates that of the 30 reported significant correlations within sex, only 4-5 of them may be false positives. For all other analyses, q-value was set to 0.05.

2.5.1 Behavioral Analysis

A total of 54 behavioral measures were selected for analysis based on relevance to addiction. Behavioral differences were assessed by behavioral cluster and goal/sign-tracking phenotype within sex using Kruskal-Wallis and Wilcoxon test. Behavioral measures significantly different by group were plotted by zscore to visualize trends between grouping phenotypes. Factor analysis was performed to test influence of weight and age at time of dissection, generation, and goal/sign-tracker phenotype using the ADONIS (PERMANOVA) function of the vegan (2.4-3) R package.

2.5.1.1 Sign-tracker/goal-tracker classification

Rats were classified as sign/goal-trackers based on PavCA Index Score (= [PavCA Score (Day 4) +PavCA Score (Day 5)]/2; see Meyer et al. 2012 for details(Meyer et al., 2012a). Subjects were classified as sign-tracker (ST) (PavCA Index Score between +0.5 and +1), goal-tracker (GT) (PavCA Index Score between -0.5 and -1), and intermediate (IN) (PavCA Index Score between -0.49 and 0.49), based on the classification method previously described (Meyer et al., 2012a).

2.5.1.2 Behavioral Cluster Analysis

All behavioral measures (total=54) were used for cluster analysis using the KODAMA package (v1.4). Behavioral data within sex was normalized using probabilistic quotient normalization and centered to zero. Data was clustered using PLS-DA (partial least squares differential analysis) grouped by goal/sign-tracking phenotype and using multiple levels of cross-validation. Entropy was tested on point values for each cross-validation level to find optimal cluster. For both the female and male behavioral data set, the lowest entropy cluster was found with PLS-DA set to parameter (f.par) 100. Hierarchical cluster analysis was performed to select optimal number of clusters and to assign samples to cluster. Results were then plotted using PCA and colored based goal/sign-tracking phenotype.

2.5.2 Microbiome analysis

Microbiome was assessed by behavioral cluster and goal/sign-tracking phenotype within sex. Kruskal-Wallis and Wilcoxon test were used to assess statistical significance in alpha diversity indices and taxonomic comparisons between groups. Beta diversity was calculated using Euclidian distance visualized and analyzed using the vegan community ecology package (v2.4-3). Adonis (PERMANOVA) function from vegan assessed beta diversity significance by generation, behavioral phenotype, sign/goal-tracker phenotype (GT, ST, IN), age and weight at time of dissection. For spearman correlations, OTUs (Operational Taxonomic Units) were filtered by median >0.01%, which resulted in sequences only present in >50% of samples and normalized to relative abundance. Correlations were performed between OTU-level bacterial abundance and behavioral measures, subset by grouping phenotype within sex. For correlation analysis, all behavioral measures were classified into 3 major categories: reward-learning, impulsivity, and locomotion (Suppl. Table 1A). For spearman correlations within sex, the qvalue R package (v2.60) was used to select false discovery rate. Due to the exploratory nature of this study, the q-value of 0.15 was accepted as it allowed reporting of interesting trends while still maintaining a low rate of false positives (4-5 total).

3. Results

3.1 Behavioral cluster analysis

We sought to evaluate the goal/sign-tracking phenotype, within sex, alongside a novel group cluster constrained by this pre-established grouping. Partial least squared differential analysis (PLS-DA) of all 54 behavioral measures (Fig. 1) within sex show a separation along the x-axis (Fig. 2A & 3A). In both males and females, the sign-tracking phenotype clusters in the negative (left) side of the x-axis (Fig. 2A & 3A). Behavioral clusters that contained the sign-tracking phenotype were labeled F.Behav.Clust.1 for female sup-population and M.Behav.Clust.1 for males. Samples clustering to the right of the x-intercept were labeled F.Behav.Clust.2 and M.Behav.Clust.2, for females and males respectively.

3.2 Behavioral differences by behavioral cluster within sex

Factor analysis of 54 behavioral measures revealed that weight at the end of the experiment was the only significant contributing factor in both females ($R2=0.036$, $p=0.028$) and males ($R2=0.037$, $p=0.018$). Goal/sign-tracker phenotype, generation, and age at time of dissection were not significant factors ($p>0.05$) contributing to variations in all behavioral measures.

All 54 behavior measures were tested within sex by goal/sign-tracker phenotype (GT, IN, ST) and behavioral cluster (Behav.Clust.1, Behav.Clust.2). Females tested by goal/sign-tracking phenotype, 15 behavioral measures were significantly different (Kruskal-Wallis, $p<0.05$), with 13 of these measures due to differences in GTs compared to STs (Wilcoxon, $p<0.01$). In males tested by goal/sign-tracking phenotype, 11 behavioral measures were significantly different (Kruskal-Wallis, $p<0.001$), with 8 of these measures due to differences in GTs compared to STs (Wilcoxon, $p<0.001$). Behavioral measure comparisons by behavioral cluster revealed 18 significant measures in females and 28 significant measures in males (Wilcoxon, $p<0.05$). Z-score was used to visualize behavioral comparisons by groups, behavioral cluster and GT/ST phenotype, within sex (Fig. 2B & 3B). See supplementary material for behavioral comparisons between sex (Supp. Section 1.2 and Fig. 1B).

*3.2.*1 *Group differences in Pavlovian Conditioned Reinforcement (CRF)*

Within both sexes and grouping phenotypes, significant differences were seen in measure for nose pokes in reinforcing port (CRF.Active.Port) and number of lever presses (CRF.Lever.Presses). In both male and female, significant differences in CRF.Active.Port were seen in goal-tracking (GT) phenotype compared to sign-tracking (ST) (Wilcoxon, $p<0.001$) and behavioral cluster grouping (Behav.Clust.1 vs. Behav.Clust.2) (Wilcoxon, $p<0.001$). Differences in CRF.Lever.Presses were explained by GT compared to ST group (Wilcoxon, $p<0.001$) and behavior cluster (Wilcoxon, $p<0.001$), in both sexes. Additionally, number of nose pokes into active port for presentation of lever reinforcer

(CRF.Number.Reinforcers) was significantly different in both male and female by GT compared to ST phenotype (Wilcoxon, $p<0.001$).

3.2.2 Group differences in Cocaine Cue Preference (CCP)

Only the male behavioral cluster showed significant differences in cocaine-induces locomotor activity in Trial 1 measured by distance traveled (CCP.T1.Cocaine.Dist) (Wilcoxon, $p<0.001$). This difference was explained by the increases in M.Behav.Clust.1 compared to M.Behav.Clust.2 (Fig. 2B).

3.2.3 Group differences in Choice Reaction Time Task (RT)

Reaction time (RT) measures of standard deviation of reaction time per epoch (RT.SDRT) and number of failed responses defined as omissions (RT.Omissions) were significantly different by female goal/sign-tracking phenotype. For both measures, this result was explained by increases in IN group compared to GT (Wilcoxon, $p<0.01$) (Fig. 3B).

3.2.4 Group differences in Locomotor Response to Novelty (Loco)

Similar to measures from CCP, M.Behav.Clust.1 had significant increases in distance travelled (Loco.Distance) compared to M.Behav.Clust.2 (Wilcoxon, $p<0.01$). This was also seen in comparisons between males behavioral cluster in measures of exploratory rearing and maximum rears (Loco.Rear and Loco.MaxRear) (Wilcoxon, $p<0.05$), locomotor activity and maximum activity (Loco.Activity and Loco.MaxAct, $p<0.05$).

3.2.5 Group differences in Light Reinforcement (LR)

Measures from the LR task were significantly different by behavioral cluster in both males and females. Total number of light onset reinforcers (LR.Reinforcers), total active and inactive responses (LR.Total), total active responses (LR.Active), and maximum active responses in an epoch (LR.Act_ Max) were significantly different (Wilcoxon, $p<0.01$). These measures were decreased in F.Behav.Clust.1 compared to F.Behav.Clust.2, while they were increased in M.Behav.Clust.1 compared to M.Behav.Clust.2 (Fig. 2B & 3B).

3.2.6 Sex differences in Delay Discounting (DD)

In female behavioral cluster, F.Behav.Clust.1 had significant increases in changes time in patch with experimenter imposed delay across epochs (DD.TIPAUC) compared to F.Behav.Clust.2 (Wilcoxon, $p<0.05$) (Fig. 3B). In the male behavioral cluster, M.Behav.Clust.1 was significantly increased in measures of patch change rate area under the curve (DD.PCRateAUC) and indifference point area under the curve (DD.IDPtAUC) compared to M.Behav.Clust.2 (Wilcoxon, $p<0.01$) (Fig. 2B).

3.3 Microbiome diversity

In Males, microbiome beta diversity was significant by generation (R2=0.039, p<0.001) and age at end of experiment (R2=0.021, p<0.001). Females were only significant by generation (R2=0.019, p<0.001). No significant differences were seen for weight, sign/goal-tracker phenotype, or behavioral cluster in either males or females.

Alpha diversity analysis by female behavioral cluster revealed significant decreases in Shannon (Wilcoxson, p<0.001) and Simpson (Wilcoxson, p<0.001) index measures in F.Behav.Clust.1 compared to F.Behav.Clust.2 (Fig. 5A & 5C). Females ST group also had significant reductions in Shannon measure of alpha diversity compared to GT (Fig. 5B) There were no significant differences in alpha diversity measures in males (Fig. 4).

3.4 OTU level bacterial differences by groups within sex

No significant differences were seen in either phenotype group by sex. In the female behavioral cluster, *Papillibacter* OTU_128 and *Blautia* OTU_160 approached significance (Wilcoxson, q=0.055). All other results had an FDR q-value greater than 0.25.

3.5 OTU level bacterial correlations to behavior

3.5.1 Correlations by Male and Female Goal/Sign-Tracker Phenotype

In males, behaviors correlated to refined OTUs by GT/ST phenotype. The strongest correlation was between cocaine induces locomotor activity during trial 4 and *Tanneralla* OTU_157 (Spearman, rho=-0.976, p<0.001). Further correlations were seen in OTU-level bacteria belonging to family Lachnispiroceae and Ruminococcaceae in measures of reward learning (LR, PavCA, RT), in reaction time and light reinforcement measures, locomotion and impulsivity measures from delay discounting (Spearman, |rho|>0.722, p<0.001). An OTU-level bacteria from genus *Thermofilum* significantly correlated to impulsivity measures only in the male intermediate (IN) phenotype (Spearman, |rho|>0.709, p<0.001). Overall, the strongest correlations were seen in the male STs (Spearman, |rho|>0.939, p<0.001) (Fig. 6A).

In females, less significant correlations between microbiome and behavior by GT/ST phenotype were seen. A total of 9 significant correlations were observed, of these 6 were significant correlations within ST phenotype. A strong trend was seen in ST females with correlations between reaction time omissions and specific OTUs assigned to genera *Vampirovibrio, Coprococcus,* and *Flavinofractor* (Spearman, rho>0.729, p<0.001). Significant correlations were also found between impulsivity

measures and OTU bacteria assigned to genera *Clostridium XIVa* and *Barnesiella* (Spearman, rho>0.682, p<0.001) (Fig. 6B).

3.5.2 Correlations by Male and Female Behavioral Cluster

No significant correlations were seen between microbiome and behavior when analyzed by male behavioral cluster (Fig. 7A).

In females, impulsivity measures and attention measures were significantly correlated with OTUs assigned to genus *Barnesiella* (Spearman, |rho|>0.620, p<0.001) (Fig. 7B).

3.5.3 Correlations by Male and Female

Spearman correlations were performed within the entire male and female sub-populations between behaviors and OTU-level bacteria (N=101, Males and Females) (Fig. 8). Impulsivity measures of indifference point area under the curve and patch change rate area under the curve was significantly correlated with *Lachnospiracaea_incertae_sedis* OTU_3469 (Impulsivity.DD.IDPtAUC and Impulsivity.DD.PCRateAUC, rho<-0.393, p<0.001) in males. In females, indifference point area under the curve and locomotor activity were negatively correlated to *Barnesiella* OTU_114 (Impulsivity.DD.IDPtAUC and Loco.Activity, rho<-0.407, p<0.001). In males, exploratory rearing was positively correlated to *Clostridium* XlVa OTU_3855; additionally, males had many OTU correlations to sensory preference for CPP floor before testing (see Supp. Materials). A positive correlation between reward learning and *Lachnospiracea incertae sedis* OTU_152 was observed in females (Reward.Learning.LR.Active, rho=0.407, p<0.001) (Fig. 8).

4 Discussion

Growing evidence suggests that understanding the complex relationship between addiction-related behaviors and microbiome is important in illuminating factors associated with addiction vulnerability. In the current study, correlations revealed novel sex-dependent links between addiction-associated behavior and microbiome. In females, operational taxonomic units (OTUs) from bacteria *Barnesiella* repeatedly correlated with behavioral measures of impulsivity, measured by indifference point area under the curve in delay discounting task (DD.IDPtAUC) (Fig. 7-8). Moreover, alpha diversity was reduced in female behavioral phenotypes associated to increased addiction vulnerability (Fig. 5). Female PLS-DA cluster phenotype (F.Behav.Clust.1 and F.Behav.Clust.2, Fig. 3A) revealed that behavioral differences followed trends similar to the goal/sign-tracking (GT/ST) phenotype. Most behavioral measures in F.Behav.Clust.1 aligned with sign-trackers (STs) and F.Behv.Clust 2 similar to goal-tracking (GTs) phenotype (Fig. 3B). In males, fewer trends were seen in both microbiome and behavior. All groups had significant correlations between OTU-level bacteria, predominantly belonging to families Ruminococcaceae and Lachnospiraceae, and behavioral measures of impulsivity, attention, reward-learning, and locomotor response to novelty.

Sign-tracking (ST) is defined as the propensity to imbue a conditioned stimulus with incentive salience (Flagel et al., 2008; Meyer et al., 2012a). STs are more responsive to both food and drug CSs than GTs and tend to engage in addiction-related behaviors. Increased ST behavior is positively correlated to increased dopamine levels in the nucleus accumbens during amphetamine self-administration (Tomie et al., 2008). Additionally, in selectively bred high/low response (HR/LR) rats, both male and females HR rats sign-track while LR counterparts goal-track. HR females have a greater propensity to self-administer cocaine than LR females and HR & LR male rats (Davis et al., 2008). In line with previous work (King et al., 2016; Pitchers et al., 2015), this study had more females assigned to the addictive-associated ST phenotype, compared to males.

There is contradictory data in humans with regard to gender differences in the incidence of addiction. However, research of both humans and other animal species indicates that females may, in some cases, display a greater propensity for addiction. A human genome-wide-association study (GWAS) showed females having greater impulsive behavior, as measured by delay discounting (Sanchez-Roige et al., 2018). Furthermore, GWAS studies suggest that opioid dependence is linked to sex specific single nucleotide polymorphisms (Yang et al., 2017). In Cloninger's typology, Type II alcoholism has an early onset, a genetic propensity, and more common in males, while Type I has a late onset and is seen in both females and males (Cloninger et al., 1996). Differences in hormones underlie some sex

differences in addiction-related behavior. Furthermore, intensity of drug usage and drug withdrawal vary depending on menstrual cycle. In the striatum, dopamine levels are higher during estrus in rats, when estradiol is elevated. Estradiol is known to increase positive affect, locomotor sensitization, and acquisition of self-administration to psychomotor stimulants in female rats (Becker et al., 2011).

Measures of impulsivity, attention, reward-learning, and locomotor response to novelty were significantly different by behavioral phenotypes and correlated to microbiome. In delay discounting, the indifference point/area under the curve measure (DD.IDPtAUC) has previously been linked to addiction in humans (Reynolds et al., 2004). This impulsivity measure correlated to OTUs assigned to family Lachnospiraceae in males and OTUs assigned to genus *Barnesiella* in females. The divergence in gut bacteria correlated to impulsivity indicates that sex-specific commensal bacteria may have similar effects on behavior. In the male intermediates (INs), measures of impulsivity from the delay discounting were significantly correlated to *Thermofilum* OTU_250 (Fig. 6). *Thermofilum* is characterized as an extremophile, thus may be exceptional in this dataset. *Thermofilum* is a sulfate respiring bacteria, and thus produces hydrogen sulfide (H2S) (Zillig et al., 1983), which has been previously been impacted in cognition and memory (Ritz et al., 2016). *Thermofilum* correlations only to male INs suggests that it has negligible associations to addiction, though may have subtle effects on attention and impulsivity. In males and females, measures of Pavlovian Conditioned Approach (PavCA) were significantly correlated with bacterial OTUs in family Ruminococcaceae. In males, reaction time (RT) measures correlated to bacterial OTUs in family Ruminococcaceae. Intriguingly, female STs revealed strong correlations between reaction time attention measure (RT.Omissions) and OTUs in family Ruminococcaceae, including genus *Flavonifrator*, and family Lachnospiraceae, as well as two OTUs in genus *Vampirovibrio*. *Vampirovibrio,* which preys on algae, and is capable of replacing taxa in the microbiome via competition (Baer and Williams, 2015; Soo et al., 2015). Significant correlations were seen in light reinforcement (LR) and locomotor response (Loco) measures and bacterial OTUs in family Ruminococcaceae, including genus *Flavonifrator*, and Lachnospiraceae in the male GT/ST phenotype. In the present study, taxonomic differences between bacterial composition in GT/IN/ST phenotypes did not reach significance. However, the observed correlations repeatedly associated to Ruminococcaceae align with previous addiction research showing reductions in Ruminococcaceae in opioid users (Acharya et al., 2017), alcohol consumption (Bull-Otterson et al., 2013; Llopis et al., 2016), and alcohol addiction severity (Leclercq et al., 2014b). Research investigating alcohol consumption and microbiome has also previously reported changes in Lachnospiraceae in mice receiving oral and vapor alcohol (Bull-Otterson et al., 2013; Peterson et al., 2017) and alcohol addiction severity in humans (Leclercq et al., 2014b). Our recently work on microbiome and addiction phenotype has illuminated intriguing trends

in bacteria from families Ruminococcaceae and Lachnospiraceae correlating to dopamine receptor expression in the striatum and measures of anxiety, novelty induced locomotor activity, impulsivity, and compulsive alcohol seeking in male rats (Jadhav et al., 2018).

Sex and/or gender are significant factors to consider when investigating the microbiome-gut-brain axis (Jasarevic et al., 2016). Previous research reveals sex-associated microbiota differences are strongly linked to metabolic processes (Amato et al., 2014; Davey et al., 2012; Markle et al., 2013; Zhernakova et al., 2016) and to influences on brain and behavior (Clarke et al., 2013; Hoban et al., 2016). Segregation of male and female rats by co-housing same sex may confound and inflate sex differences observed especially in light of coprophagic behavior of rats; this environmental factor may potentially influence microbiota more than host genetics (Rothschild et al., 2018). Although, in a study of howler monkeys microbiome continued to be distinct between sex despite shared environment and parent (Amato et al., 2014). Clearly, further investigation is necessary to elucidate the connections between sex and microbiome composition as well as factors such as genotype (Palmer and de Wit, 2012) and cagemates (Baud et al., 2017).

Females associated to a ST phenotype had decreased alpha diversity compared to GTs (Fig. 5). No difference was seen in alpha diversity between GT/IN/ST phenotypes in males (Fig. 4). Reduced alpha diversity is generally attributed to poor health, with reductions similarly reported in mental health conditions including stress and depression (Bailey et al., 2011; Kelly et al., 2016). Reduced alpha diversity has also been shown in chronic-intermittent vapor ethanol exposure (Peterson et al., 2017) and antibiotic depletion is linked to increased locomotor response to cocaine (Kiraly et al., 2016). Alterations in Ruminococcaceae and Lachnospiraceae have been characterized in many health conditions. Importantly, reduced intestinal permeability, liver damage, and inflammation (Acharya et al., 2017; Bailey et al., 2011; Bajaj et al., 2012; Bull-Otterson et al., 2013; Leclercq et al., 2014a; Leclercq et al., 2017; Llopis et al., 2016; Petrov et al., 2017) have been implicated to alterations in these two family-level bacteria in conditions of psychological stress (Bangsgaard Bendtsen et al., 2012; Dunphy-Doherty et al., 2018), depression (Chen et al., 2018; Jiang et al., 2015; Naseribafrouei et al., 2014), autism (Golubeva et al., 2017; Hsiao et al., 2013), and dopaminergic-mediated disorders (Leclercq et al., 2014b; Petrov et al., 2017). Many novel bacteria were identified in this study in relation to addictive phenotype and microbiome. OTUs associated to genus *Flavonifractor* were correlated to locomotor activity in males and RT omissions in females. Increased abundance of *Flavonifractor* is linked to major depressive disorder (Jiang et al., 2015) and bipolar disease (Coello et al., 2018); the mechanism of how *Flavonifractor* effects brain is not well known, though it is believed to cause oxidative stress and

inflammation. Furthermore, OTUs associated to *Barnesiella* were repeatedly correlated to behaviors in females, particularly impulsivity measures. In a human study comparing microbiota composition of alcohol drinkers and non-drinkers, a top differentiated OTU increased with alcohol consumption belonged to an uncharacterized Barnesiella (Kosnicki et al., 2018). Genus *Barnesiella* has also previously been characterized as preventing colonization of infection bacteria (Steinway et al., 2015; Ubeda et al., 2013), thus reducing inflammatory profile in the gastrointestinal tract. In this study, we see two novel taxa which have been characterized as replacing taxa in the microbiome, *Barnesiella* and *Vampirovibrio.* These novel taxa were also related to measures of attention and impulsivity. Previous research in humans reported a negative relationship between *Clostridium XIVa* and depression in females (Chen et al., 2018). Further investigation is necessary to determine if these identified bacteria preferentially colonize certain sexes, the mechanism for the preference, in addition to how these certain bacteria are influencing brain and behavior. Furthermore, the impact of immune function must be investigated in these behavioral phenotypes associated with specific bacteria (Rea et al., 2016). Research has shown that gut bacteria impact neuroimmunology (Braniste et al., 2014; Scott et al., 2017), and neuroimmunology has been linked to addiction (Hofford et al., 2018). Future studies should also examine how the neurobiological substrates of the behavioral changes observed are regulated by the microbiome. Indeed, the gut microbiome has been shown to regulate cortical morphology and neurotransmitter expression, notably in the prefrontal cortex which is involved in impulsivity behaviors (Hoban et al., 2017; Hoban et al., 2016; Luczynski et al., 2016). Moreover, investigations into the role of the vagus nerve as a conduit of signals from the gut to the brain is also warranted (Bravo et al., 2011).

Conclusion

This is the first time, to our knowledge, that extensive characterization of within sex addiction-phenotype and behavioral measures have been associated to microbiome. The most robust findings in this study indicate that microbiome is associated to locomotor response, reward-stimulus learning, impulsivity and attention. Notably, impulsivity measure was repeatedly correlated to certain bacteria in males and females. This novel work impresses facts that sex as a factor must be considered in both behavior and microbiome research. Further investigation is necessary to elucidate factors that contribute to sex differences in microbiome, and how these differences influence other addiction-related measures like drug-self administration and relapse.

Conflicts of interest

JFC & TGD are in receipt of research funding from 4D-Pharma, Mead Johnson, Suntory Wellness, Nutricia, and Cremo. Timothy Dinan has been an invited speaker at meetings organized by Servier,

Lundbeck, Janssen, and AstraZeneca. John Cryan has been an invited speaker at meetings organized by Mead Johnsen, Alkermes, and Janssen.

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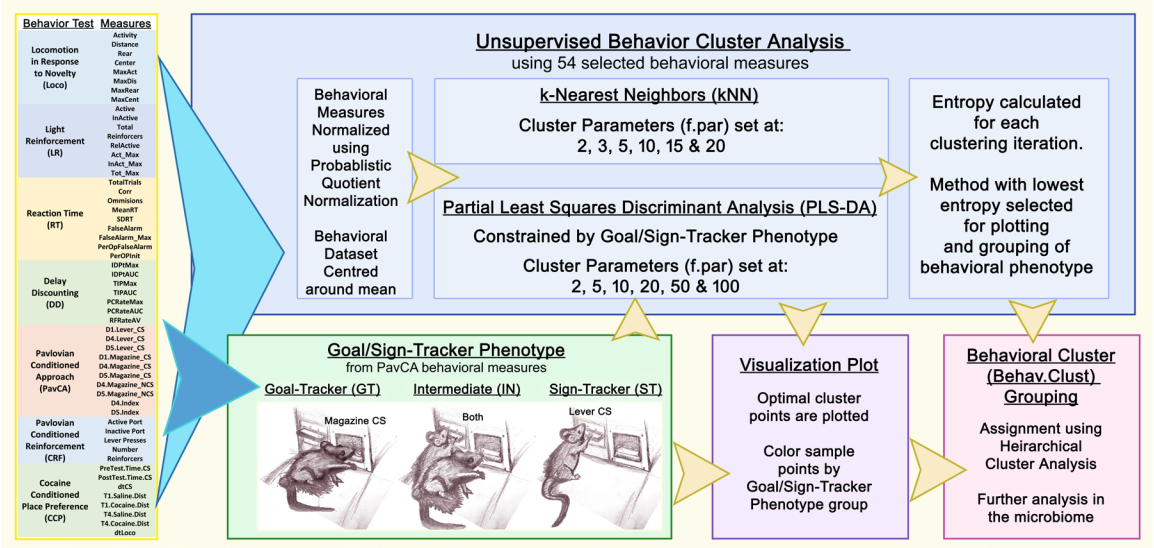

Fig 1 – Flow Diagram of how behavioral measures are used to create goal/sign-tracking phenotype and behavioral cluster phenotype.

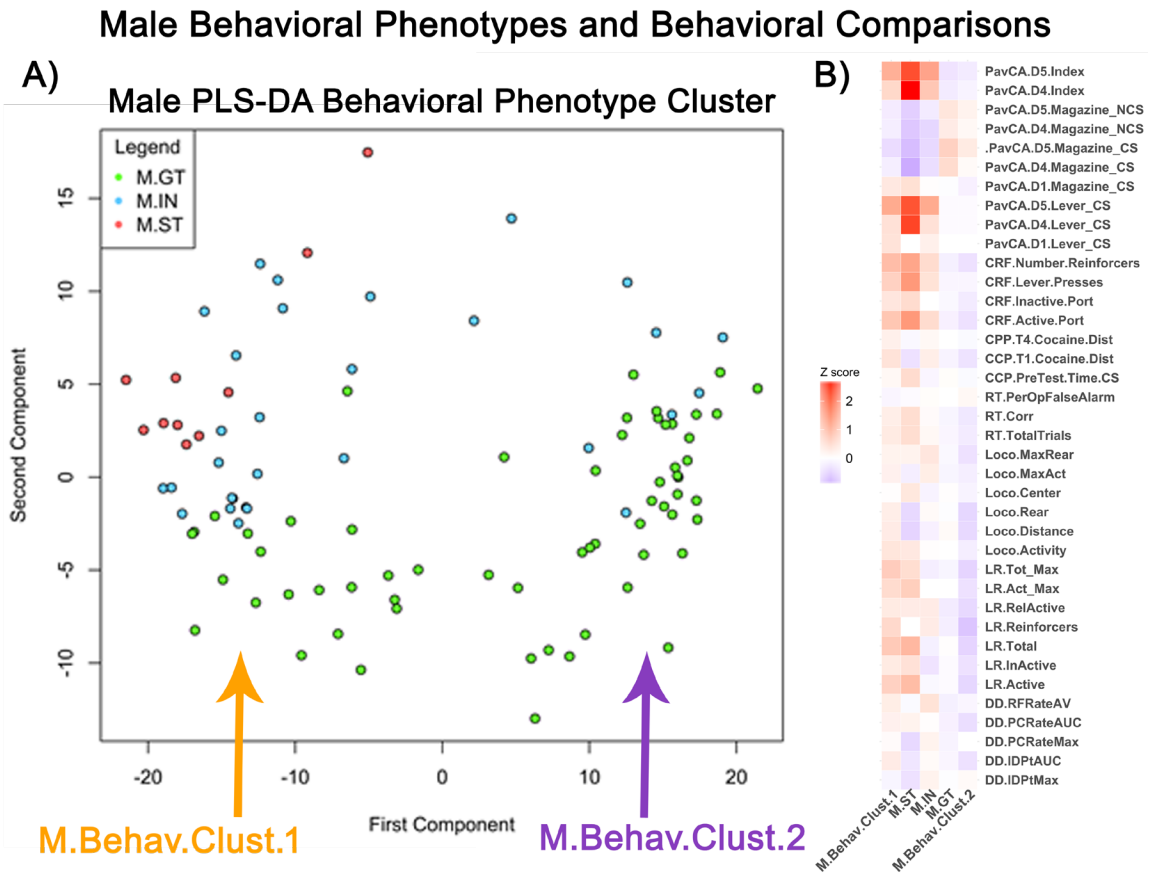

Figure 2 – Male Behavioral Phenotypes and Behavioral Comparisons – A) Behavioral Phenotype Cluster visualized in principal coordinate analysis (PCA) of PLS-DA clustered behavioral measures. Each dot represents an individual rat, distance from one dot to another represents overall differences in behavioral measures.Goal-trackers (GT) colored green, intermediate (IN) blue, sign-trackers (ST) red. B) Z score indicate increases (red) or decreases (blue) in behavioral measures by behavioral cluster group and goal/sign-tracker phenotype group compared to entire male population.

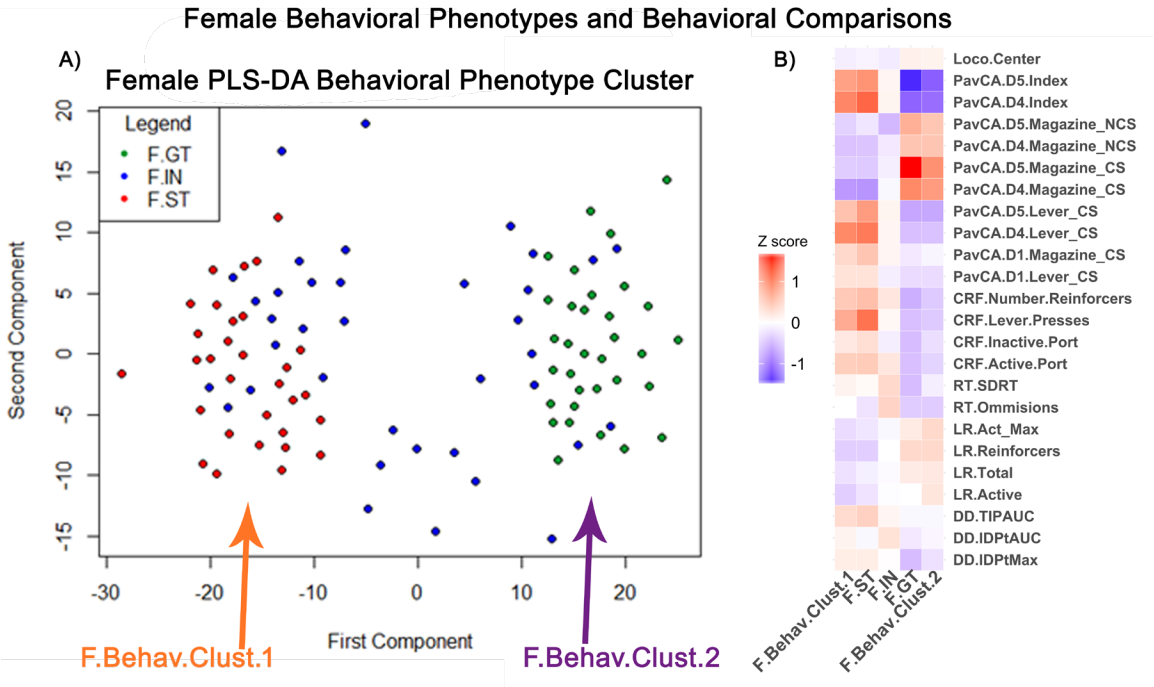

Figure 3 – Female Behavioral Phenotypes and Behavioral Comparisons – A) Behavioral Phenotype Cluster visualized in principal coordinate analysis (PCA) of PLS-DA clustered behavioral measures. Each dot represents an individual rat, distance from one dot to another represents overall differences in behavioral measures.Goal-trackers (GT) colored green, intermediate (IN) blue, sign-trackers (ST) red. B) Z score indicate increases (red) or decreases (blue) in behavioral measures by behavioral cluster group and goal/sign-tracker phenotype group compared to entire female population.

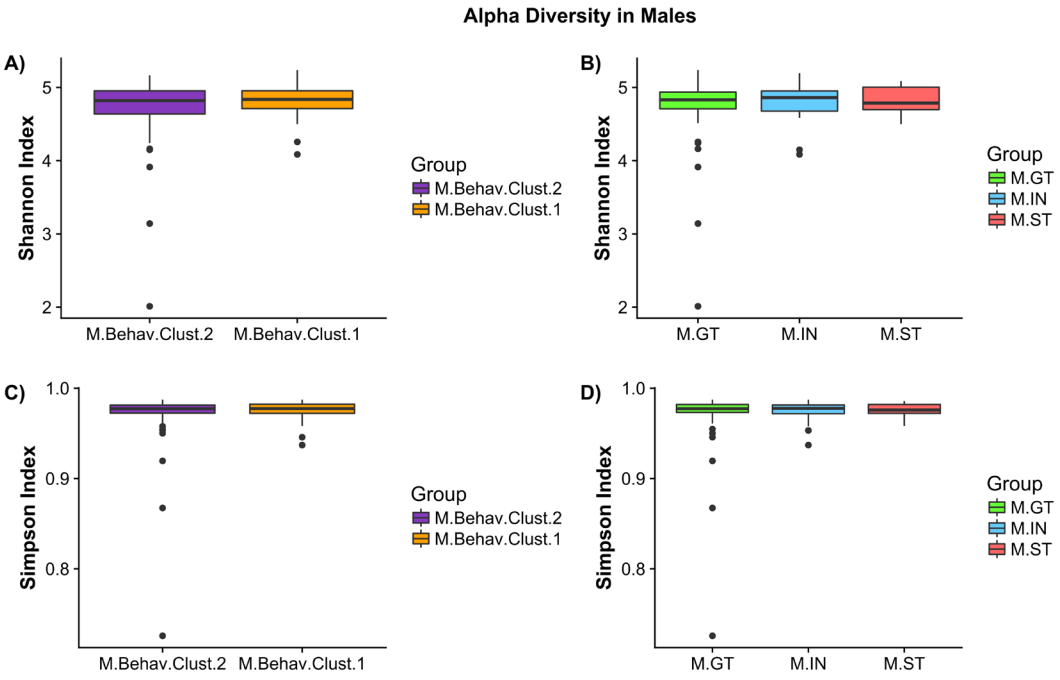

Figure 4 – Male Alpha Diversity by Phenotype Group – A) Shannon index measure of alpha diversity by behavioral cluster (M.Behav.Clust 1 = orange, M.Behav.Clust.2 = purple). B) Shannon index by goal/sign-tracking phenotype: goal-tracker (M.GT = green), intermediate (M.IN = blue), and sign-tracker (M.ST = red). C) Simpson index measures of male alpha diversity by behavioral cluster. D) Simpson index of male goal/sign-tracker phenotype.

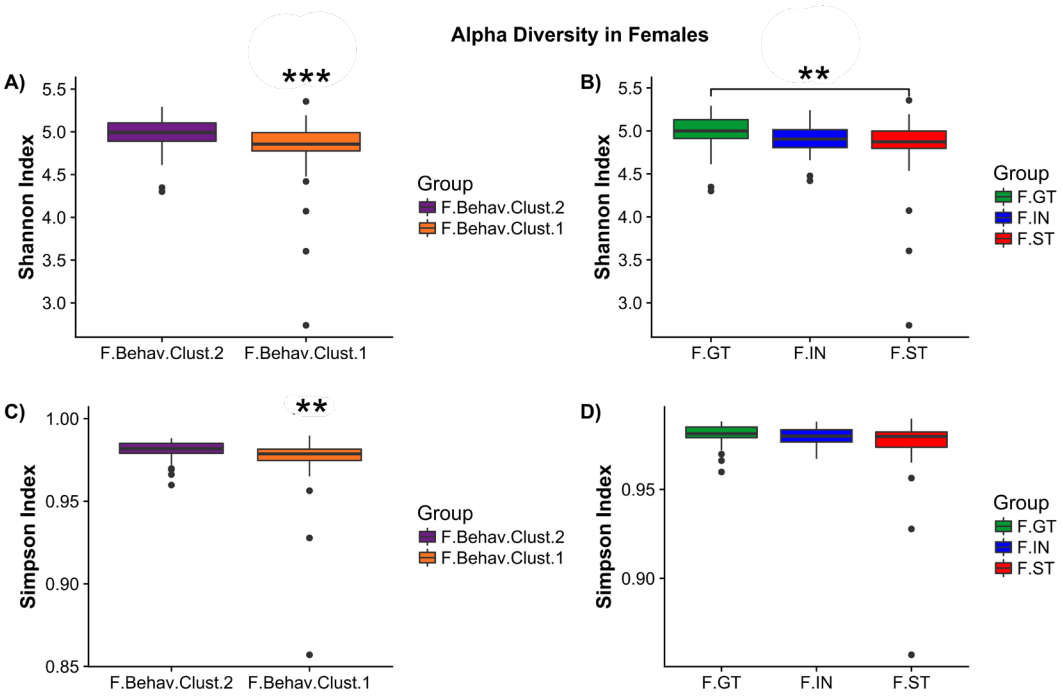

Figure 5 – Female Alpha Diversity by Phenotype Group – A) Shannon index measure of alpha diversity by behavioral cluster (F.Behav.Clust 1 = orange, F.Behav.Clust.2 = purple). B) Shannon index by goal/sign-tracking phenotype: goal-tracker (F.GT = green), intermediate (F.IN = blue), and sign-tracker (F.ST = red). C) Simpson index measures of female alpha diversity by behavioral cluster. D) Simpson index of female goal/sign-tracker phenotype. Asterisks indicate significance: '***' $p<0.001$, '**' $p<0.01$

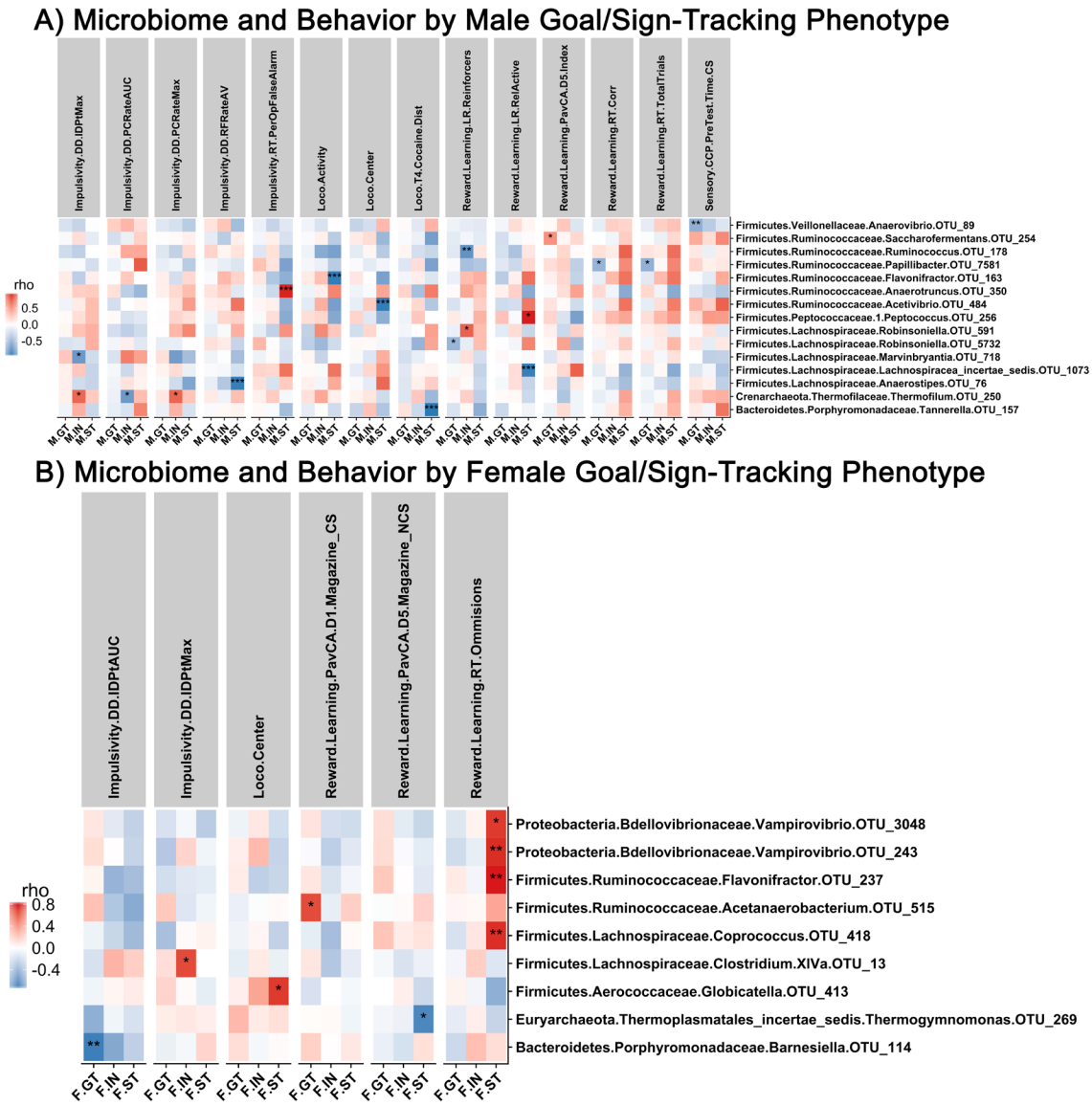

Figure 6 – Correlation Analysis in Male and Female Goal/Sign-Tracking Phenotype. A) Correlations between OTU-level bacteria and behavior in male goal/sign-tracking phenotype. B) Correlations between OTU-level bacteria and behavior in female goal/sign-tracking phenotype. Positive correlations indicated in red, negative correlations indicated in blue. Significance that passes FDR indicated by asterisk: '***' q<0.05, '**' q<0.10, '*' q<0.15

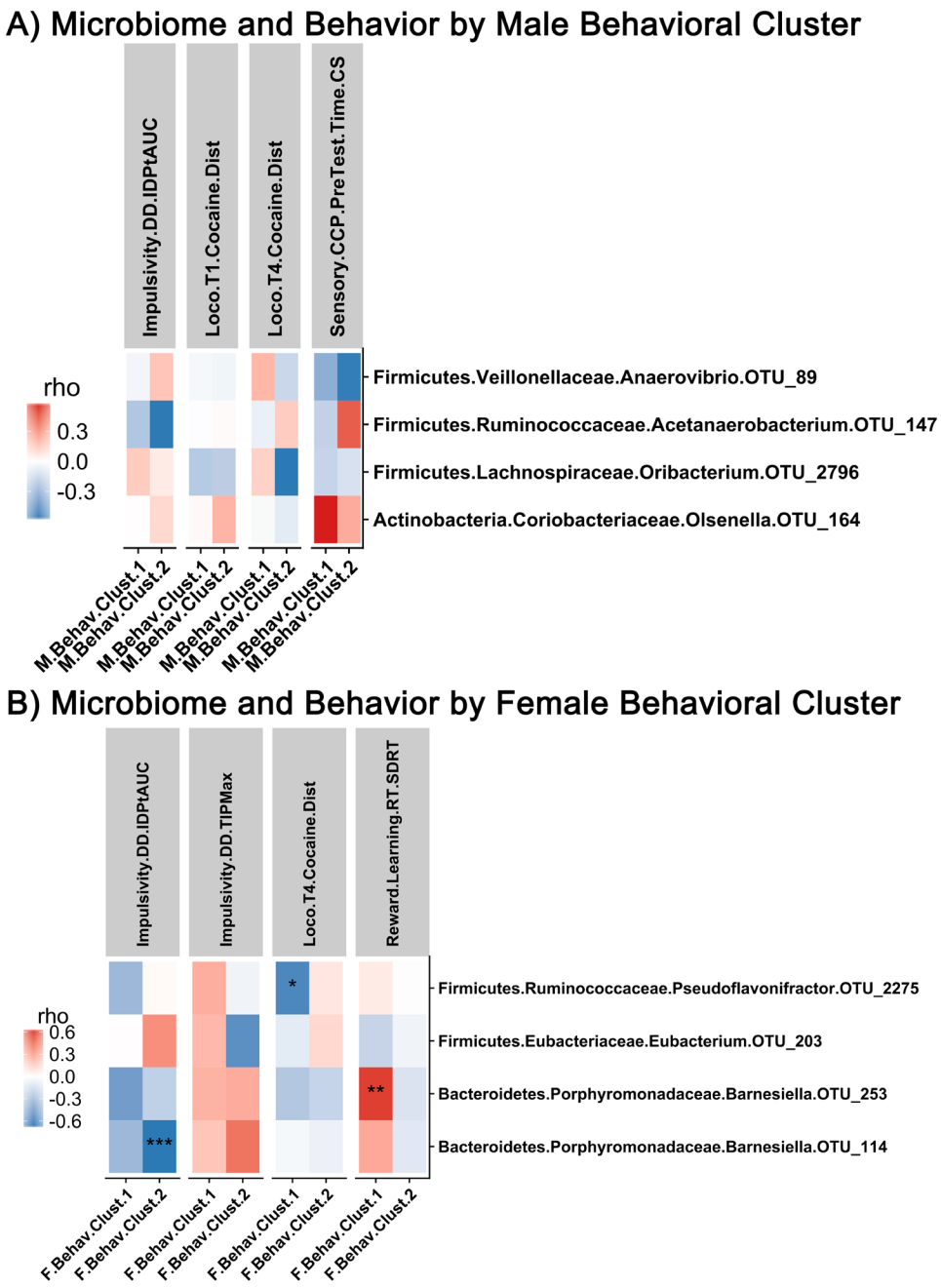

Figure 7 – Correlation Analysis in Male and Female Behavioral Clusters. A) Correlation between OTU-level bacteria and behavior in male behavioral cluster phenotype. B) Correlation between OTU-level bacteria and behavior in female behavioral cluster phenotype. Positive correlations indicated in red, negative correlations indicated in blue. Significance that passes FDR indicated by asterisk: '***' q<0.05, '**' q<0.10, '*' q<0.15

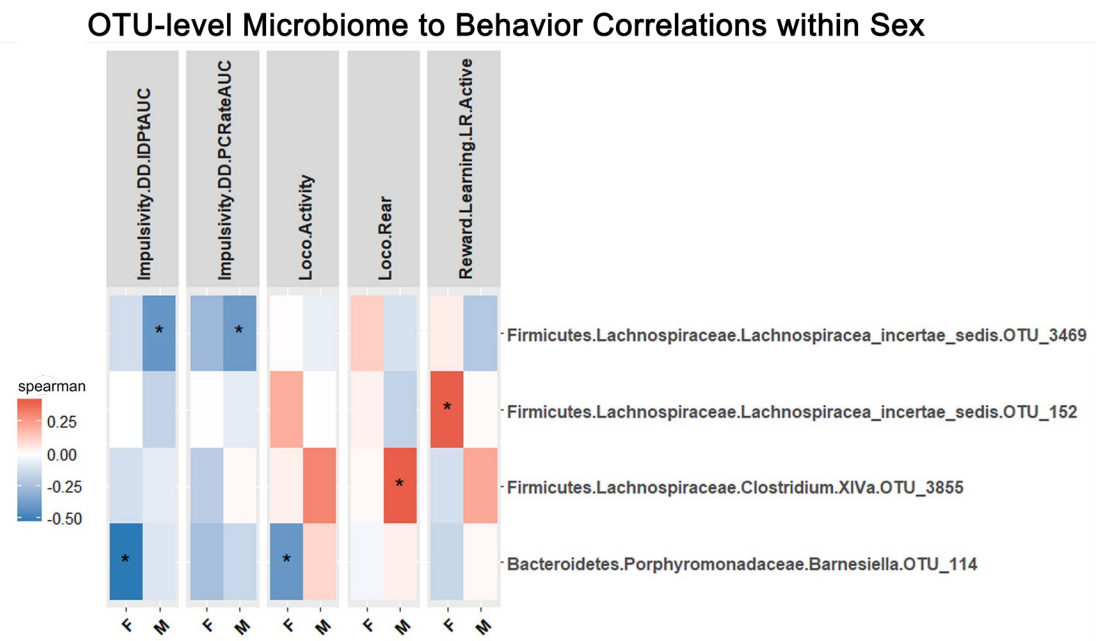

Figure 8 – Correlation Analysis by sex. Correlations between OTU-level bacteria and behavior in entire female and male populations. Positive correlations indicated in red, negative correlations indicated in blue. Significance that passes FDR indicated by asterisk: '***' q<0.05, '**' q<0.10, '*' q<0.15

Age	Procedure
0-21	Rats reared in Medical College of Wisconson
21	Rats arrive in Buffalo, NY (21 days old).
60	Rats housed until they are young adults (60 days of age)
63	Locomotor Response to Novelty
86	Light Reinforcement
129	Choice Reaction Time Task
154	Delay Discounting
171	Pavlovian Conditioned Approach (1+5 days), then Conditioned Reinforcement (1 day)
185	Cocaine Cue Preference
200	Rats sacrificed; cecal samples collected

Table 1: Study design flow chart – Age in days (first column), Procedure (second column).

Sex, Drugs, and the Microbiome: Microbiota Composition Correlates with an Addiction-Associated Behavioral Phenotype in a Sex-Dependent Manner

or

Sex, Drugs, and the Microbiome: Goal/Sign-Tracking Phenotype Reveals Associations between Behavior and Microbiome in a Sex-Dependent Manner in the Rat

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Supplementary documentation

1. Preliminary results of Sex Differences (Male vs Female)

1.1 Behavioral cluster analysis

Partial least squares differential analysis (PLS-DA) of all 54 behavioral measures constrained by sex and goal/sign tracking (GT/ST) phenotype resulted in a separation between males and females (Fig. S1A). 81% of males are present in the left cluster (L.Behav.Clust), while only 26% of females are present in the L.Behav.Clust. In the right behavioral phenotype cluster (R.Behav.Clust), 74% of females are present while only 19% of males are present. Interestingly, the majority of female and male sign-trackers (ST) congregated in the lower right quadrant of this cluster.

1.2 Behavioral differences between sex and behavioral cluster

Factor analysis (PERMANOVA) of 54 behavioral measures revealed that sex was the only significant contributing factor ($R^2=0.301$, $p=0.0002$). To ensure this observation was not due to sex differences in weight, factor analysis was also performed for animal weights at experiment endpoint. Weight was not a significant factor contributing to behavior ($R^2=0.006$, $p=0.173$). Additionally, sign/goal tracker phenotype was not a significant factor for all behavioral measures ($R^2=0.007$, $p=0.371$). Of 54 behaviors tested, 32 were significantly different when compared by sex, 34 between left and right behavioral cluster, 17 when females only where compared by behavioral cluster, and 20 when males only were compared by behavioral cluster. Z-score was used to represent behavioral differences by sex, behavioral cluster (R.Behav.Clust and L.Behav.Clust), and behavioral cluster by sex (Fig S1B).

1.2.1 Sex differences in Pavlovian Conditioned Approach (PavCA)

Females had significantly increased sign-tracking behavior compared to males (Fig. S1A-B). This was evident by significant increases in Pavlovian Index score on day 4 (PavCA.D4.Index, $p<0.0001$) and day 5 (PavCA.D5.Index, $p<0.01$) in females compared to males. Pavlovian Index score was also significant between behavior cluster (PavCA.D4.Index and PavCA.D5.Index, $p<0.001$) and when males and females were compared individually by behavioral cluster (PavCA.D4.Index and PavCA.D5.Index,

p<0.001). Sign-tracking behavior, as measured by lever-cue associations, was significantly increased in females, R.Behav.Clust, and R.Behav,Clust.F on day 1, 4, and 5 (PavCA.D1.Lever_CS, p<0.001; PavCA.D4.Lever_CS, p<0.001; PavCA.D5.Lever_CS, p<0.001). Male right behavioral cluster (R.Behav.Clust) was also significantly increased on day 4 and 5 (PavCA.D4.Lever_CS, p<0.001; PavCA.D5.Lever_CS, p<0.001) compared to left behavioral male cluster (L.Behav.Clust). These finding show that sign-tracking behavior is increased in all females compared to males, as well as right and left behavioral cluster, either compared with all samples or within sex.

1.2.2 Sex differences in Pavlovian Conditioned Reinforcement (CRF)

Females had significant increases in reinforcement behavior compared to males (Fig. S1B). Number of CS lever presentations was significantly different between sex, behavioral cluster, and behavioral cluster by sex (CRF.Number.Reinforcers, p<0.001), and total number of lever presses (CRF.LeverPresses, p<0.001). These measures were significantly increased in females vs. males. L.Behav.Clust vs. R.Behav.Clust, and left behavioral clusters within both sexes (Fig. S1B).

1.2.3 Sex differences in Cocaine Cue Preference (CCP)

Females had an overall significant increase in locomotor distance travelled compared to males following cocaine administration at both trial 1 and 4 (CCP.T1.Cocaine.Dist and CCP.T4.Cocaine.Dist, p< 0.001) (Fig. S1B). Significant differences in cocaine-induces locomotor travel was also observed between behavioral clusters, however when behavioral clusters were compared within sex, only significant differences were seen within females at trial 1 (CCP.T1.Cocaine.Dist, p< 0.01). Behavioral cluster comparisons in all samples (L.Behav.Clust vs. R.Behav.Clust) showed significant differences in locomotor behavior between cocaine administration and saline (CCP.dtLoco, p<0.01).

There were no significant sex differences in the primary measures of change in time (s) in cocaine-paired (CS+) floor between the post-test and pre-test (CCP.dtCS, p>0.05).

1.2.4 Sex differences in Choice Reaction Time Task (RT)

Reaction time (RT) measures of impulsivity total number of per opportunity false alarms was not significant (RT.POFA, p>0.05). A significant increase in total number of per opportunity

premature initiations (RT.PerOPInit, $p<0.001$) was observed in females compared to males and left behavioral cluster compared to right (Fig. S1B).

1.2.5 Sex differences in Locomotor Response to Novelty (Loco)

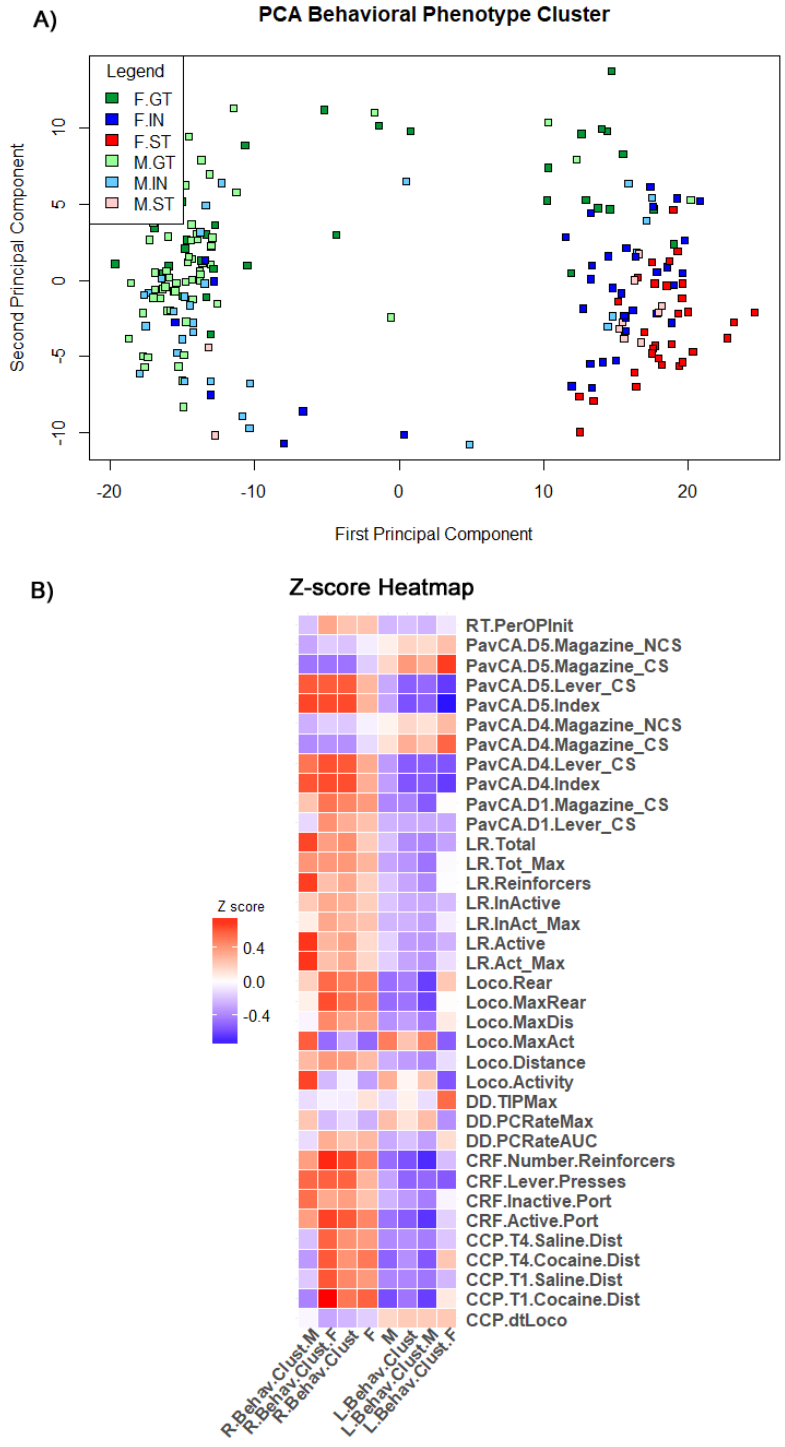
Similar to measures from CCP, females had significant increases in distance travelled (Loco.Distance, $p<0.001$). This was also seen in comparisons between behavioral cluster and within the male behavioral cluster (Loco.Distance, $p<0.05$). Additionally, there was significant increases in maximum exploratory rearing measures in females, R.Behav.Clust, R.Behav.Clust.F and R.Behav.Clust.M (Loco.MaxRear, $p<0.01$). Significant decreases in total locomotor activity (Loco.Activity, $p<0.001$) in females compared to males.

1.2.6 Sex differences in Light Reinforcement (LR)

Total Light Reinforcement LR.Reinforcers, $p<0.01$) and total inactive responses (LR.InActive,$p<0.05$) were significantly different by sex, behavioral cluster, and within the male behavioral cluster. Significant increases in measures total active and inactive responses in females, R.Behav.Clust, R.Behav.Clust.F and R.Behav.Clust.M (LR.Total , $p<0.01$) were observed (Fig. S1B).

1.2.7 Sex differences in Delay Discounting (DD)

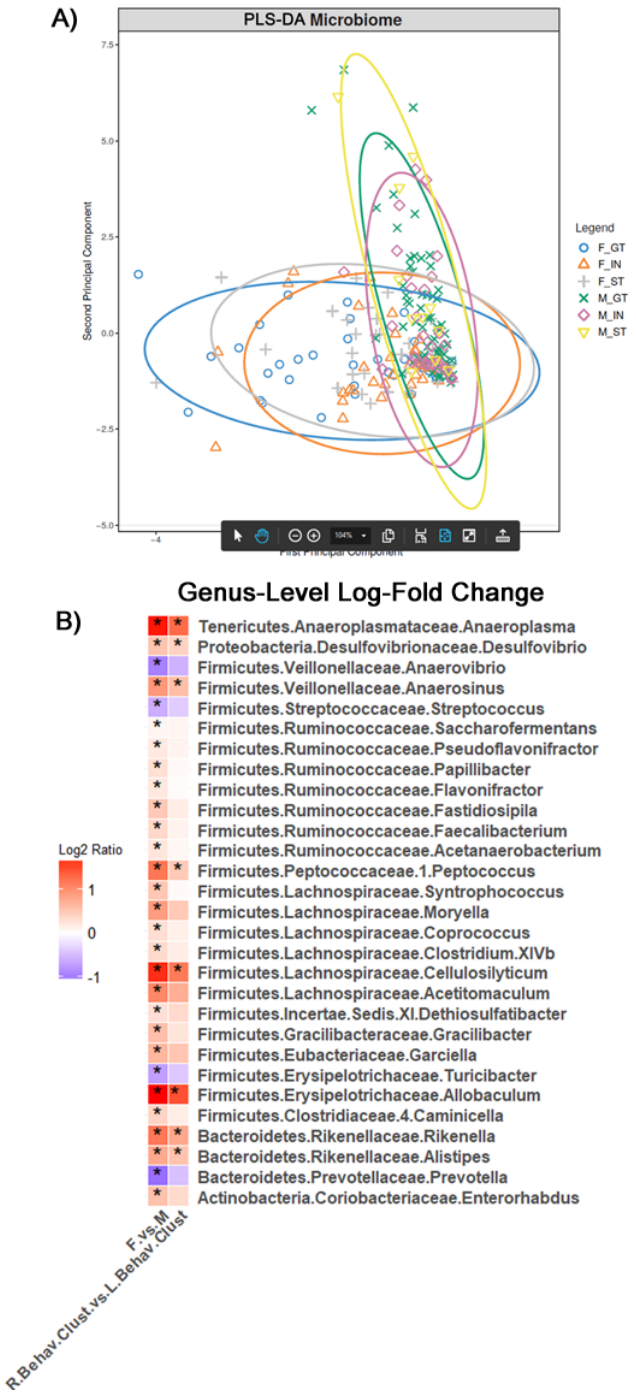
Females had significant increases in impulsivity measure of patch change rate area under the curve (DD.PCRateAUC, $p<0.01$). This was also seen in comparisons between behavioral cluster ($p<0.05$). Primary measures of indifference point area under the curve was not significantly (DD.IDPtAUC, $p>0.05$).



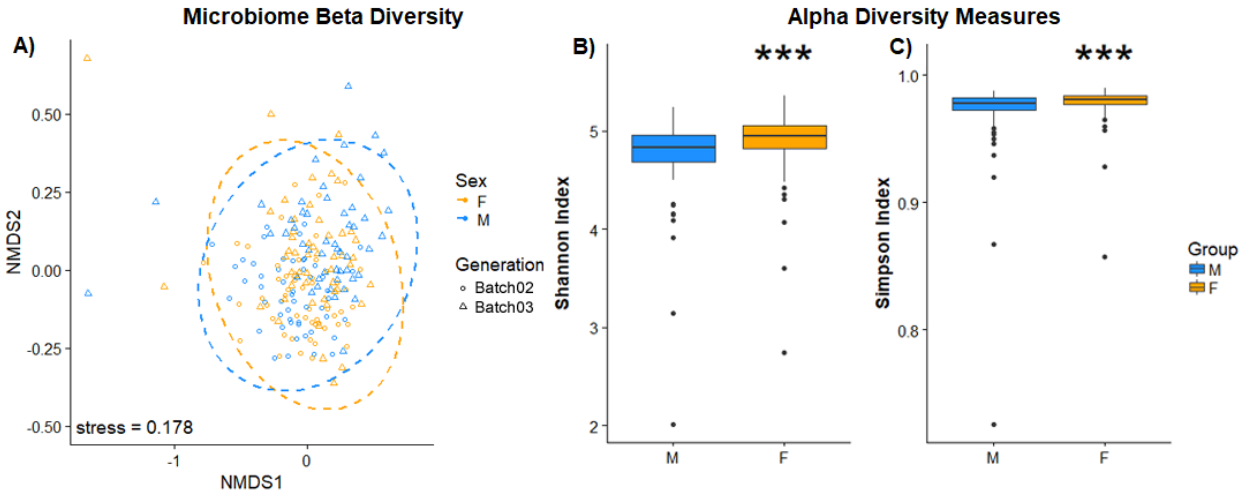
Supplementary Figure 1 – Behavioral differences – A) Behavioral Phenotype Cluster visualized in principal coordinate analysis (PCA) of PLS-DA clustered behavioral measures. Goal-trackers (GT) colored green, intermediate (IN) blue, sign-trackers (ST) red; females in dark colors, and males light colors. B) Z score indicate increases (red) or decreases (blue) in behavioral measures compared to population.

1.3 Microbiome diversity

Microbiome beta diversity was significantly different by sex (R2=0.016, p=0.002) and generation (R2=0.032, p=0.0002) (Fig. S3A). Age was also a significant factor contributing to microbiome variance (R2=0.017, p=0.001). No significant differences were found for weight (R2=0.005, p=0.312) or sign/goal-tracker phenotype (R2=0.011, p=0.285). OTU assignments of sign/goal-tracker phenotype only clustered by sex in PLS-DA (Fig 2A). Alpha diversity analysis by sex revealed significant differences in Shannon (p=0.0001) and Simpson (p=0.001) index measures (Fig. S2B). There were no significant differences in alpha diversity measures between behavioral cluster within sex (p>0.05).



Supplementary Figure 2 – Genus Differences in Microbiome by Sex and Behavioral Phenotype – A) PCA beta diversity plot of PLS-DA clustered microbiome by sign/goal-tracker phenotype and sex (blue circle = female goal-tracker (F_GT), orange triangle = female intermediate (F_IN), grey plus = female sign-tracker (F_ST), green x = male goal-tracker (M_GT),purple diamond = male intermediate (M_IN), yellow triangle = male sign-tracker (M_ST). B) Log-fold change differences at genus level between females vs males and behavioral phenotype clusters (R.Behav.Clust vs. L.Behav.Clust); asterisks indicates significance after FDR corrections (p>0.05)



Supplementary Figure 3 – Microbiome Diversity – A) NMDS plot of beta diversity by sex (Female = yellow, Male = blue) and by generation (circles = Batch02, and triangles = Batch03); stress value in lower left corner indicates how well the data is representation in reduced the dimensions. B & C) Alpha diversity of B) Shannon and C) Simpson index measures between males (blue) and females (yellow); asterisks indicate significance p>0.001.

1.4 Taxonomic differences between sex

At the phylum level, Tenericutes was significantly greater in females compared to males and (p<0.001) (Fig. S2B). Among females and R.Behav.Clust, significantly greater proportions of Peptococcaceae (p<0.001) and the corresponding genus *Peptococcus* (p<0.001), Rikenellaceae (p<0.001) and the genera *Alistipes* (p=0.01) and *Rikenella* (p<0.001), Desulfovibrionaceae (p<0.01) and the genus *Desulfovibrio* (p<0.01), and in family Erysipelotrichaceae (p>0.05) genus *Allobaculum* (p<0.01). In females compared to males, greater abundance of Gracilibacteraceae (p=1.24e-4) and the genus *Gracilibacter* (p=1.24e-4), as well as Enterobacteriaceae (p=0.002). Significantly lower Streptococcaceae (p=0.002) and the genus *Streptococcus* (p=0.004) were observed (Fig. S2B). Other significant differences were found at the genus level, including several genera from the families Lachnospiracea, Ruminococcaceae, Veillonellaceae, and Anaeroplasmataceae (Fig. S2B).

1.5 Microbiome correlations to Behavior

Spearman correlations were performed on all 54 behavioral measures and OTU-level bacterial abundance filtered at >0.01% median abundance, presence greater >50% all samples. For all samples qvalue=0.05, within sex qvalue=0.15.

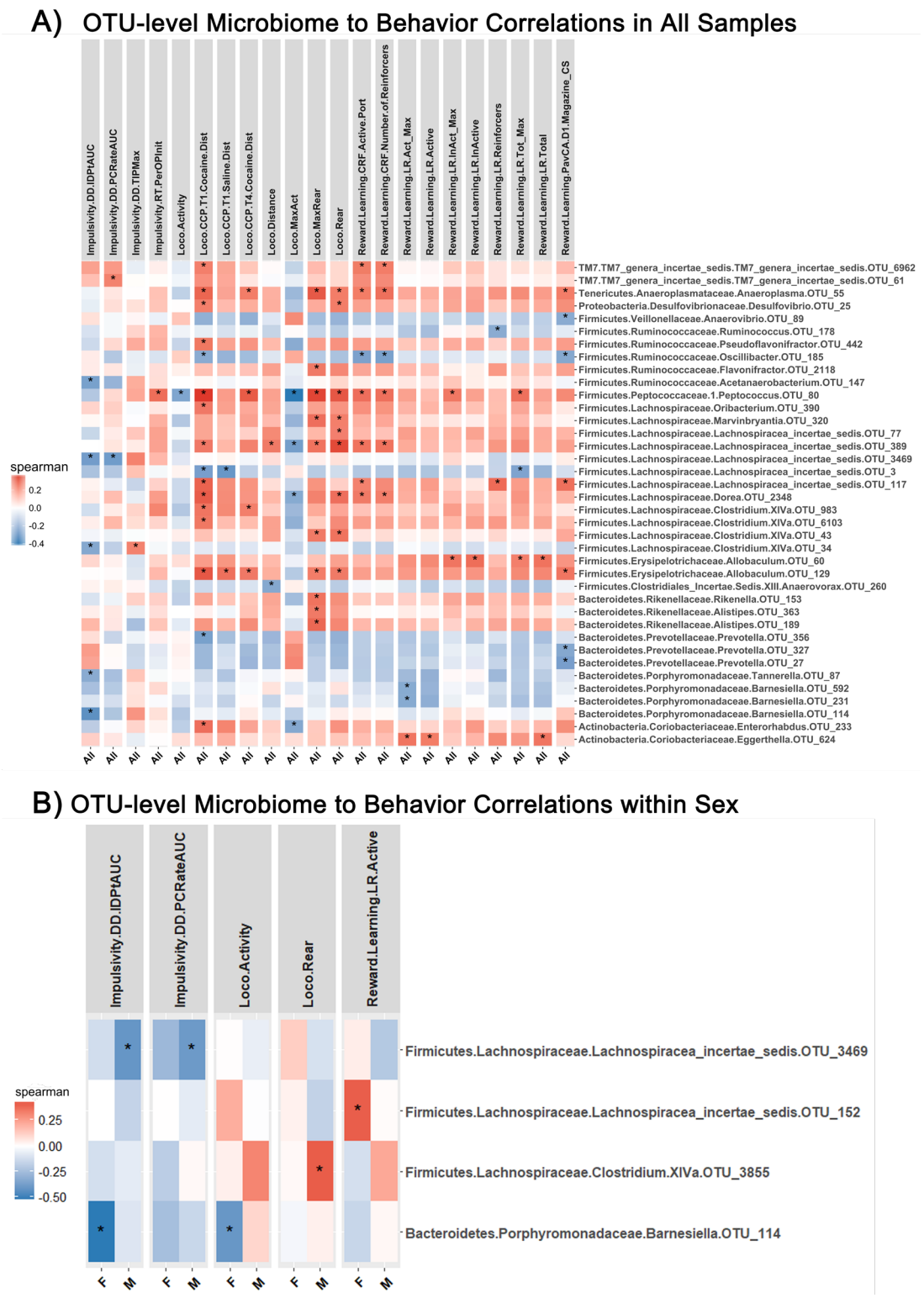
1.5.1 Correlations to Behavioral Measures in All Samples

Spearman correlations were performed between behaviors and OTU-level bacteria for all samples (N=202) (Fig. S4A). In all samples, significantly correlations were positively correlated to *Anaeroplasma* OTU_55*, Peptococcus* OTU_80*, Allobaculum* OTU_60 and OTU_129, and *Lachnospiraceae_incertae_sedis* OTU_117, *Allobaculum* OTU_129 were positively correlated to goal tracking behavior on day 1 (Reward.Learning.PavCA.D1.Magazine_CS, rho>0.262, p<0.001); *Anaerovirbrio* OTU_89, *Oscillibacter* OTU_185, and *Prevotella* OTU_327 and OTU_27 were negatively correlated to in goal tracking behavior on day 1 (Reward.Learning.PavCA.D1.Magazine_CS, rho<-0.254, p<0.001). Distance travelled following cocaine administration during trial 1 and 4 was positively correlated to *Allobaculum* OTU_129, *Anaeroplasma* OTU_55, *Peptococcus* OTU_80, *Clostridium XIVa* OTU_983, among others (Loco.CCP.T[1or4]. CocaineDist, rho>0.2.55, p<0.001). Total number of presentations of lever (Reward.Learning.CRF.Number.of.Reinforcers, rho>0.280, p<0.001), and maximum exploratory rears per epoch (Loco.MaxRear, rho>0.282, p<0.0001) were positively correlated to *Anaeroplasma* OTU_55, *Peptococcus* OTU_80*,* and *Lachnospiracea_incertae_sedis* OTU_389. Significant negative correlations to impulsivity measures of indifference point area under the curve and patch change rate area under the curve (Impulsivity.DD.IDPtAUC and Impulsivity.DD.PCRateAUC, rho<-0.303, p<0.001) was observed with *Lachnospiracea_incertae_sedis* OTU_3469. Correlations to locomotor activity (Loco.Activity) and other locomotor response to novelty measures were significantly (p<0.05) associated to *Peptococcus* OTU_80, *Lachnospiracaea_incertae_sedis* OTU_389, and *Allobaculum* OTU_129. All reported rho and p values can be found in Supp. Material.

1.5.2 Correlations to Behavioral Measures within Sex

Spearman correlations were performed between behaviors and OTU-level bacteria within sex (N=101, Males and Females) (Fig. S4B). Impulsivity measures of indifference point area under the curve and patch change rate area under the curve was significantly correlated to *Lachnospiracaea_incertae_sedis* OTU_3469 (Impulsivity.DD.IDPtAUC and Impulsivity.DD.PCRateAUC, rho<-0.393, p<0.001) in males. In females, indifference point area under the curve and locomotor activity were negatively correlated to *Barnesiella* OTU_114 (Impulsivity.DD.IDPtAUC and Loco.Activity, rho<-0.407, p<0.001). In males, exploratory rearing was positively correlated to *Clostridium* XlVa OTU_3855; additionally, males had many OTU correlations to sensory preference for CPP floor before testing (see Supp. Materials). A positive correlation between reward learning and *Lachnospiracea*

incertae sedis OTU_152 was seen in females (Reward.Learning.LR.Active, rho=0.407, p=2.44e-05) (Fig. S4B).



Supplementary Figure 4 – Correlation Analysis. A) Correlations between OUT-level bacteria and behavior in all subjects. Significance that passes FDR indicates by asterisk ($q<0.05$) B) Correlation between OTU-level bacteria within sex and behavior. Significance that passes FDR indicates by asterisk ($q<0.15$). Positive correlations indicated in red, negative correlations indicated in blue.

1.5.3 Correlations to Behavioral Phenotype Cluster X-axis and OTUs

Behavioral Phenotype Cluster (Fig. S1A) separated into two clusters along the X-axis. These X-axis values were correlated to OTU abundance. For all samples qvalue=0.05, within sex qvalue=0.15.

1.5.3.1 Correlations to Behavioral Phenotype Cluster X-axis and OTUs in All samples

In all samples, X-axis behavioral phenotype cluster values positively correlated to *Peptococcus* OTU_80, *Anaeroplasma* OTU_55, *Allobaculum* OTU_33 and OTU_129, *Lachnospiracaeae_incertae_sedis* OTU_389, *Dorea* OTU_2348, and *Acetivibrio* OTU_586 (rho>0.241, p<0.001) (Table S1). X-axis phenotype cluster was negatively correlated to *Anaerostipes* OTU_5419, *Tannerella* OTU_1263, *Barnesiella* OTU_871 and OTU_726, and *Lachnospiracea incertae sedis* OTU_3 (rho<-0.240, p<0.001) (Table S1).

1.5.3.2 Correlations to Behavioral Phenotype Cluster X-axis and OTUs within Sex

In males, X-axis behavioral phenotype cluster values positively correlated to alpha diversity measures (Observed and Chao1, rho=0.236, p<0.5). In females, X-axis values behavioral phenotype cluster (Fig. S1A) negatively correlated to Shannon index alpha diversity measure and *Barnesiella* OTU_253 (rho<-0.231, p<0.05).

OTU	rho	Pvalue
Firmicutes.Peptococcaceae 1.Peptococcus.OTU_80	0.34596	4.58E-07
Tenericutes.Anaeroplasmataceae.Anaeroplasma.OTU_55	0.324795	2.40E-06
Firmicutes.Erysipelotrichaceae.Allobaculum.OTU_33	0.308244	8.08E-06
Firmicutes.Lachnospiraceae.Lachnospiracea_incertae_sedis.OTU_389	0.276757	6.69E-05
Firmicutes.Erysipelotrichaceae.Allobaculum.OTU_129	0.265397	0.000135
Firmicutes.Lachnospiraceae.Anaerostipes.OTU_5419	-0.25981	0.000188
Bacteroidetes.Porphyromonadaceae.Tannerella.OTU_1263	-0.25524	0.000246
Bacteroidetes.Porphyromonadaceae.Barnesiella.OTU_871	-0.2534	0.000274
Firmicutes.Lachnospiraceae.Dorea.OTU_2348	0.247933	0.000374
Firmicutes.Lachnospiraceae.Lachnospiracea_incertae_sedis.OTU_3	-0.24325	0.000486
Firmicutes.Ruminococcaceae.Acetivibrio.OTU_586	0.241062	0.000548
Bacteroidetes.Porphyromonadaceae.Barnesiella.OTU_726	-0.24049	0.000566

Supplementary Table 2: OTU Correlations to Behavioral Cluster x-axis in all samples – OTU correlations to x-axis correlations in behavioral cluster (Figure 1A) that pass FDR corrections (Q<0.05). OUT (first column), rho value (spearman, second column), Pvalue (raw, third column)