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Gut microbiome correlates with altered striatal dopamine receptor expression in a model of compulsive alcohol seeking

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Highlights

- Less than 15% of rats display diagnostic criteria of uncontrolled alcohol seeking
- These vulnerable rats have reduced striatal dopamine 2 receptor expression
- And increased striatal dopamine 1 receptor expression
- Significant correlations were observed between microbiome and dopamine receptors

Keywords

Alcohol addiction, microbiome, gut-brain axis, alcohol use disorder, impulsivity

Abstract

Identifying biological markers predicting vulnerability to develop excessive alcohol consumption may lead to a real improvement of clinical care. With converging evidence suggesting that gut microbiome is capable of influencing brain and behavior, this study aimed at investigating whether changes in gut microbiome composition is associated with conditioned responses to alcohol. We trained Wistar rats to self-administer alcohol for a prolonged period before screening those exhibiting uncontrolled alcohol seeking and taking by modeling diagnostic criteria for AUD: inability to abstain during a signaled period of reward unavailability, increased motivation assessed in a progressive effortful task and persistent alcohol intake despite aversive foot shocks. Based on addiction criteria scores, rats were assigned to either Vulnerable or Resilient groups. Vulnerable rats not only displayed increased impulsive and compulsive behaviors, but also displayed increased relapse after abstinence and increased sensitivity to baclofen treatments compared to resilient animals. Then, rats underwent a 3-month wash out period before sacrifice. Dorsal striatum was collected to assess dopamine receptor mRNA expression, and 16S microbiome sequencing was performed on caecal contents. Multiple significant correlations were found between gut microbiome and impulsivity measures, as well as augmentations in striatal Dopamine 1 receptor (D1R) and reductions in D2R as vulnerability to AUD increased. Therefore, using a singular translational approach based on biobehavioral dispositions to excessive alcohol seeking without heavy intoxication, our observations suggests an association between gut microbiome composition and these specific “at risk” behavioral traits observed in our translationally relevant model.

1. Introduction

Unhealthy alcohol use is one of the world's leading causes of death and diseases. Recent reports underline that approximately 3.3 million deaths worldwide and 139 million disability adjusted life years are attributed to alcohol use (World Health Organization, 2014). Besides this unacceptable human cost, alcohol use disorder (AUD) represents a growing economic burden worldwide, total cost of which is estimated in the range of US$ 200-700 billion annually, both in Europe and in the United States (Barrio et al., 2017; Baumberg, 2006; Sacks et al., 2015). Reduction of heavy drinking and relapse prevention currently represent the main therapeutic objectives in the treatment of alcohol use disorder, but the ratio of good responders remains much too low to be satisfying (Mann and Hermann, 2010). Therefore, alternative approaches should be privileged. One strategy would consist of better identifying "problem drinkers" in the general population who are not yet manifesting major symptoms of heavy intoxication but are drinking at levels that increase risks for medical and psychosocial consequences (Saitz, 2009). Growing evidence points out to a role of the microbiota-gut-brain axis in AUD, with excessive ethanol consumption altering the gut microbiome, increasing the intestinal permeability and exacerbating systemic inflammation, ultimately amplifying comorbidities classically observed in alcoholic patients (Bull-Otterson et al., 2013; de Timary et al., 2015; Gorky and Schwaber, 2016; Leclercq et al., 2017, 2014a, 2014b, 2012; Temko et al., 2017).

Recent observations have begun to shed light on the inextricable connection between microbes and mammals, leading to the provocative postulate that humans would not have developed the current level of cognitive performance in absence of bacteria (Cryan and Dinan, 2012; Dinan et al., 2015; Montiel-Castro et al., 2013;

Sampson and Mazmanian, 2015). In support of this assertion, a current consensus has now established how the commensal microbiota of the intestine greatly influence all aspects of physiology, including a fine tuning of brain function and behavior (Doherty et al., 2017; Hoban et al., 2017). With the intestinal microbiome collectively encoding more than 3.3 million of non-redundant genes (Qin et al., 2010), exceeding by far the number encoded by the human host genome, large-scale metagenomic projects have endeavored to unveil the contribution of gut microbes to the unconscious system regulating behavior. In this perspective, there is a growing appreciation of the role of the gut microbiome in regulating brain and behavior, in health and disease (Dinan and Cryan, 2017, Sherwin et al, 2017). Overall, the microbiota–gut–brain axis helps maintaining homeostasis of the brain by controlling central physiological processes including neurotransmission, neurogenesis, neuroinflammation and neuroendocrine signaling (Clarke et al., 2014).

In particular, recent reports suggest that alcohol exposure triggers neuroimmune and inflammatory processes in the brain (Crews et al., 2017a, 2017b, 2013). Although the source of this neuroinflammation is not yet understood, growing evidence suggests that alterations in microbiota composition may contribute to neuroimmune processes and peripheral inflammation (de Timary et al., 2015; Gorky and Schwaber, 2016; Rea et al., 2016). Changes in the gut microbiome have been reported in both human alcoholic individuals and murine models of chronic alcohol exposure, with increased intestinal permeability (causing endotoxin to escape into the circulation and impact the host), increased abundance of pro-inflammatory gut microbes, like Proteobacteria species, and decreased abundance of normal commensal bacteria like Bacteroidetes (Bull-Otterson et al., 2013; Leclercq et al., 2017; Mutlu et al., 2012; Peterson et al., 2017).

However, if chronic excessive alcohol use seems to significantly impact the microbiota, it does not seem to be sufficient to cause gut dysfunction in all alcohol dependent patients, since altered microbiota composition was reported in only a subset of alcoholics. Further, alterations in microbial composition were not correlated to the duration of sobriety, suggesting alcohol-related dysbiosis is long-lasting and persists despite abstinent periods (Leclercq et al., 2017, 2014a, 2014b, 2012; Mutlu et al., 2012). Meanwhile, gut microbial and peripheral metabolite level alterations remain narrowly linked to alcohol craving, anxiety, and depression, considered important personality traits associated with the vulnerability to develop AUD (de Timary et al., 2015; Leclercq et al., 2014a, 2014b, 2012).

The inability to control drug taking in general, and conditioned responses in particular, is a complex brain disorder that affects the most vulnerable individuals and worsens with recurring drug consumption. Therefore, understanding the heterogeneity in the behavioral characteristic of patients with AUD is warranted for developing personalized treatments. We recently claimed that most preclinical studies still defend pharmacology-centered views that do not really capture the inter-individual vulnerability to lose control over alcohol consumption (Jadhav et al., 2017). Considering that preclinical investigations about genetic/temperament predisposition to alcohol abuse require the development of an appropriate and relevant animal model, we adapted to rodents a few criteria used for screening AUD according to the DSM and contributed to recognize that addiction is a progressive and idiosyncratic disorder. The recent reports suggesting that personality traits associated with risk for drug addiction may be linked to the microbiome-gut-brain axis (Bravo et al., 2011; Collins et al., 2013; Golubeva et al., 2017; McVey Neufeld et al., 2016) calls for further investigation to determine whether

gut microbiome diversity and composition may be associated with behaviors related to alcohol use disorders.

To this end, we assessed microbiota composition was different in rats exhibiting a biobehavioral disposition to lose control over alcohol consumption by comparison with resilient animals. Moreover, we sought to investigate of changes in the microbiome correlated with alterations in striatal dopamine receptor levels that may underlie the observed behavioural changes in the rat model of AUD.

2. Material and methods

2.1. Animals

Male Wistar rats were bred in-house at the Center for Psychiatric Neuroscience animal facility (breeders ordered from Charles River, France). They were approximately 7 weeks old and weighed 200–250 grams at the beginning of the experiment. They were kept in reversed 12-h light/dark cycle (lights off at 8.30 am) and housed in controlled temperature and humidity conditions. All experiments were performed in accordance with the Swiss Federal Act on Animal Protection and the Swiss Animal Ordinance and were approved by the cantonal veterinary office (authorization 3047 to B.B).

2.2. Behavioral phenotyping of alcohol use disorder

The procedure for screening addiction-like behaviors has been extensively described elsewhere (Jadhav et al., 2017). Briefly, rats were first monitored for assessing impulsive behaviors using a 5-choice serial reaction time task paradigm (5-CSRTT).

They were then tested in an elevated plus maze for measuring their anxiety-like behaviors. Then, rats were daily trained for 30 min to self-administer 0.1 ml of alcohol 10% weight/volume for 80 consecutive sessions, before being screened for addiction-like behavior (see supplementary information for further detail). Test sessions aiming at identifying rats at risk of losing control over alcohol intake operationalized 3 diagnostic criteria for AUD: inability to abstain during a signaled period of reward unavailability, increased motivation assessed in a progressive effortful task and persistent alcohol intake despite aversive foot shocks. Each rat was considered positive for one addiction-like criterion if its score reached the 66th-99th percentile of the total distribution. The addiction score was calculated as the sum of the standardized scores of each of the addiction-like criteria (Deroche-gamonet et al., 2004). A total of 60 rats were trained (1 outlier exhibiting very high lever pressing behavior was excluded), with those identified as positive for 2-3 criteria, defined as Vulnerable were grouped together (N=19), and those with 0 and 1 criterion grouped and named Resilient (N=40). To further validate our model, rats were first exposed to a conflict situation in which they had to bear electrical foot shocks prior to get access to ethanol. Second, we tested baclofen responses in both groups of rats in order to assess whether the reinforcing and motivational properties of alcohol in resilient and vulnerable rats were differentially sensitive to an anti-alcohol effect treatment. Further details on the behavioral procedure are provided in Supplementary Information.

2.3. Ceacal microbiome collection and sequencing

All samples from the Vulnerable group (N=19) and an equivalent subset from Resilient group (N=19) were used for microbiome analysis. The selected Vulnerable rats

belonged to the top 33% of the population for the three behaviors and the Resilient rats belonged to the lowest 33% of the population. Caecum was collected following three months of abstinence under aseptic conditions and snap-frozen on dry ice. Protocols for microbiome sequencing were used as previously described (Peterson et al., 2017). Briefly, caecal contents from frozen caecum (stored at -80ºC) was extracted under a sterile hood. The QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract bacterial DNA from caecal contents using the manufacturer's handbook (Second Edition 2012) *Isolation of DNA from Stool for Pathogen Detection* protocol. 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'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGN GGCWGCAG; Reverse:5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACT ACHVGGGTATCTAATCC) (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 (Klindworth et al., 2013). 16S rRNA amplicons were sequenced on the Illumina MiSeq platform (Teagasc, Morrepark, Ireland).

2.4. Microbiome Sequence Processing

Sequence reads in FASTQ files were joined using FLASH 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) using USEARCH (Version 7.0-64bit). The average number of high-quality sequences generated per sample was 150,707 ± 69,666 SD. Taxonomy was assigned to OTUs using Silva version 123. Alpha diversity indices were calculated with Qiime.

2.5. Gene expression analysis

Samples from 0Crit (N=8) and 3Crit (N=7) group were selected for gene expression analysis. Whole brains were extracted and rapidly sliced into 2 mm-thick coronal sections in a rat brain stainless steel matrix. Slices containing dorsal striatum were used for micro-punch dissection (0.98 mm diameter micro-punch, Stoelting, Dublin, Ireland). RNA was extracted with a RNeasy Plus Minikit (Qiagen, Valencia, CA, USA) and converted into cDNA by reverse transcription reaction using TaqMan Reverse Transcriptase Reagents (Applied Biosystem, Foster City, CA, USA). Real-time PCR amplification was performed with an ABIPRISM 7500 cycler and SYBER green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) using specific sets of primers (Microsynth AG, 9436 Balgach, Switzerland). Forward and reverse primers for the tested genes are the following: β-actin = forward: 5′-GCTTCTTTGCAGCTCCTTCGT-3′, reverse: 5′-ATATCGTCATCCATGGCGAAC-3′; D1 receptor = forward: 5'-GGAGGACACCGAGGATGA-3', reverse: 5'-ATGAGGGACGATGAAATGG-3', D2 receptor = forward: 5'-TGGGTCAGAAGGGAAGG-3', reverse: 5'-GATGATAAAGATGAGGAGGGT-3'. All samples were analyzed in triplicates. Relative gene expression was measured with the comparative ΔΔCt method24 and normalized with β-actin transcript levels.

2.6. Statistical Analysis

For behavioral & mRNA Analysis, data was tested for normality (Shapiro-Wilks test) and equality of variances (Levene's Test). Data are expressed as mean ± standard error (SE). Parametric data were analyzed by one way- and two-way ANOVAs followed by Bonferroni corrections, respectively. Homoscedastic, parametric measures were evaluated with two sample T-test. Unpaired T test was used to analyze the anxiety data. Nonparametric measures were evaluated with Mann–Whitney test. Baclofen response was calculated with a Wilcoxon sign-ranks test. The level of significance was set at 0.05, and analyses were performed using IBM SPSS Statistics 23.

Microbiota analysis was performed in R (v3.3.3) and RStudio (v1.0.136). Plots were generated in R using ggplot2 package (v2.2.1). Mann–Whitney test was used to assess statistical significance in alpha diversity indices and taxonomic comparisons between groups. Beta diversity was visualized and analyzed by OTU counts normalized using the wisconsin function from vegan community ecology package (v2.4-3). Adonis (PERMANOVA, permutations=999) vegan function assessed beta diversity significance between groups. Spearman correlation was performed on genus and family level bacterial abundance, behavioral measures, and dopamine receptor mRNA relative-expression levels. Since dopamine mRNA expression data was only available for 15 subjects, correlations to microbiome only included these 15 subjects. In all other behaviour correlations, all samples were used (N=38). All correlations and taxonomic comparisons were corrected for multiple testing using the q value (v2.6.0) R package, with the critical value for false discovery rate (Q) set at 0.10. Log2 fold ratio calculated mean genus-level change in abundance for the Vulnerable group relative to Resilient group.

3. Results

3.1. Identification of rats at risk of alcohol use disorder

After 80 sessions of operant conditioning (fixed ratio 1, time out 4 sec to get 0.1 mL of 10% w/v ethanol, Figure 1A), rats underwent a procedure for screening evidence for addiction-like behavior. A rat was considered positive for an addiction-like criterion when its score was in the 66th to 99th percentile of the distribution. Hence, of the total 59 rats, we obtained 4 groups, 26 rats with 0 criterion, 14 rats with 1 criterion, 12 rats with 2 criteria and 7 rats with 3 criteria (Figure 1B).

One-way ANOVAs revealed significant differences in the inability to abstain during a signaled period of reward unavailability ($F_{3,55} = 17.436$, $p < 0.0001$, Figure 1C), in the motivation to seek for ethanol in a progressive effortful task ($F_{3,55} = 23.23$, $p < 0.0001$, Figure 1D), in the persistence in ethanol seeking despite aversive foot shocks ($F_{3,55} = 22.55$, $p < 0.0001$, Figure 1E), and finally in the vulnerability to relapse after a period of abstinence ($F_{3,55}=6.13$, $p=0.0012$, Figure 1G). Further statistical analyses are provided in Supplementary Information.

The addiction scores, calculated as the sum of the standardized scores of each of the addiction-like criteria, were significantly different from each other ($F_{3,55} = 67.20$, $p<0.0001$), and were linearly increasing from 0crit to 3crit rats (Figure 1 F). We therefore clubbed 0 and 1 crit rats together and named them Resilient, while 2 and 3 crit rats were grouped and named Vulnerable.

Further statistical analyses (available in the Supplementary Information) showed increased compulsive behavior and increased sensitivity to baclofen treatments (Figure 1 H, I and J), as well as increased predisposing impulsivity (Figure 1K) in Vulnerable

rats compared to Resilient ones. Ultimately, factor analysis revealed that the three addiction-like criteria, the reinstatement and the pre-existing trait of impulsivity loaded on one construct accounting for 50% of the variance, and therefore measuring one single underlying factor. Overall, these series of observations served as a strong rationale for identifying rats with a loss of control-prone phenotype, without heavy ethanol intoxication given their history of brief exposures to alcohol used in this procedure.

3.2. Behavioral profiling of selected resilient and vulnerable rats

Nineteen Resilient (eighteen 0Crit and one 1Crit rats) and nineteen Vulnerable (twelve 2Crit and seven 3Crit rats) animals among the 59 rats initially screened were selected for microbiome analyses (see supplementary information). A brief presentation of their respective behaviors is summarized on Figure 2.

Vulnerable rats exhibited increased alcohol seeking behaviors, in the presence of shock (Mann–Whitney U = 14, $p<0.001$, Figure 2A) and absence of ethanol (Mann–Whitney U = 15, $p<0.001$, Figure 2B), and increased motivation assessed in a progressive ratio schedule of reinforcement (Mann–Whitney U = 11, $p<0.001$, Figure 2C). Addiction score and reinstatement of a lever pressing behavior after a period of abstinence were significantly higher in Vulnerable rats compared to Resilient (Mann–Whitney U = 0, $p<0.001$, Figure 2D and T-Test $t(36)=-4.50$, $p<0.001$, Figure 2E, respectively). Vulnerable rats' response to baclofen treatment (1mg/kg) was enhanced, with a breaking point for ethanol seeking significantly reduced compared to resilient rats (Mann–Whitney $Z=-2.24$, $p<0.05$, Figure 2F).

Vulnerable rats exhibited enhanced motor impulsivity, reflected by the percentage of premature responses in a 5-choice serial-reaction time task (T-test $t(36)=-2.74$, $p<0.01$, Figure 3A). They did not display any anxiety-like behavior on the elevated plus maze (Mann–Whitney $U = 136$, $p>0.05$, Figure 3B), but increased novelty induced locomotor activity (Mann–Whitney $U = 99$, $p<0.05$, Figure 3C) compared to resilient animals. Total ethanol consumed over the entire 80 self-administration sessions was analyzed for subjects used in microbiome analysis. There was no significant difference between groups ($t(36)=-1.73$, $p>0.05$). Although the Vulnerable group had significantly higher body weight compared to Resilient ($t(36)=-2.38$, $p<0.05$), cecum weight was not significantly different between groups ($t(36)=0.40$, $p>0.05$).

Finally, given the importance of the dopaminergic system in the striatum and the pivotal role it plays in the reward circuitry, we investigated the expression of the D1 receptor and D2 receptor in the dorsal striatum. D1 receptor expression was significantly higher ($t(13)=-2.88$, $p<0.05$) and D2 receptor expression significantly lower in the Vulnerable group ($t(13)=5.54$, $p<0.001$) compared to Resilient group (Figure 3E-F).

3.3. 16S microbiome analyses in caecal contents of resilient and vulnerable rats

3.3.1. Alpha and Beta Diversity

Alpha and beta diversity analysis revealed no significant difference between Vulnerable and Resilient group, but a trend towards increased richness and evenness in the Vulnerable group (Figure 4A-B).

3.3.2. Taxa Level Relative Abundance

Compositional comparisons at the phylum, family, and genus level showed no significant difference between Vulnerable and Resilient group after False Discovery Rate (FDR) correction ($p>0.05$). At phylum level, a trend towards increased Firmicutes and decreased Actinobacteria in Vulnerable group ($p>0.05$) were seen. Comparisons at the family level revealed trends of increased Ruminococcaceae and decreased Bacillales Family.XI and Deferribacteraceae in Vunerable group. Additionally, changes in many genera of Lachnospiraceae and Ruminococcaceae were observed, however did not pass FDR significance testing ($p>0.05$) (Figure 5).

3.3.3. Microbiome, Behaviour, and mRNA expression Correlation Analysis

The strongest correlations were seen between D2R mRNA expression and low abundance bacteria belonging to phylum Firmicutes ($|rho|>0.55$, $p<0.05$), comprised mainly of positive correlations. The largest decrease in Vulnerable relative to Resilient group was observed in genus *Veillonella* (log2 ratio=-4.6), which was negatively correlated to D1R mRNA expression ($rho<-0.58$, $p<0.05$). Other significant correlations to D1R mRNA expression include genera *Gemella* ($rho<-0.61$, $p<0.05$), and two from family Ruminococcaceae ($rho<-0.57$, $p<0.05$) (Figure 6).

AUD behaviour showed a significate positive correlation to bacteria in order Clostridiales ($rho>0.35$, $p<0.05$), including many genera from family Ruminococcaceae and Lachnospiraceae. Significant, negative correlations were seen between AUD behaviour and genera *Desulfovibrio* ($rho<-0.45$, $p<0.01$), *Gemella* ($rho<-0.43$, $p<0.01$), uncultured Coriobacteriaceae ($rho<-0.40$, $p<0.05$), and *Hydrogenoanaerobacterium* ($rho<-0.36$, $p<0.05$). The 5-Choice Serial Reaction Time Task measure of impulsivity

showed significant positive correlations to two genera of *Lachnospiraceae* (rho>0.34, p<0.05), *Lachnospiraceae uncultured* and *Lachnospiraceae UCG-005*, and significant negative correlation to bacteria in family Ruminococcoceae (rho<-0.35, p<0.05), an uncultured bacterium in the *Eubacterium coprostanoligenes* group. Anxiety measures (percentage time on open arms) from the EPM test were significantly positively correlated to the genus level bacteria *Lachnospiraceae UCG-006* (rho>0.41, p<0.01) and *Papillibacter* (rho>0.34, p<0.05), in family Ruminococcoceae. On the other hand, EPM anxiety measure was negatively correlated to genus *Anaerofilum* (rho<-0.42, p<0.01), in family Ruminococcoceae. OFT measure of novelty induced locomotion negatively correlated to genera *Lachnospiraceae UCG-007* (rho<-0.44, p<0.01) and an uncultured bacterium in the *Ruminococcoceae Eubacterium coprostanoligenes* group (rho<-0.40, p<0.05). Total ethanol (EtOH) consumption (TotEtOH) significantly correlated with *Gemella* (rho<-0.37, p<0.05) (Figure 6).

Most correlations are seen in low abundance bacteria (mean abundance < 0.001%) however most of these low abundance genus level bacteria are present in the majority of samples (Supplementary Table 4). At the family level, Bacillales Family XI is most frequently correlated to behaviour measures. Both at the genus and family level this bacterium is in very low abundance and only present in 9 of the 38 samples. Family level correlations, relative abundance and presence in samples of bacteria correlating to addiction measures is listed in Supplementary Tables 3-6.

4. Discussion

A link between alterations in microbiota and alcohol-related behavioural changes has remained relatively unexplored. Here we show for what is to our knowledge the first time microbiota composition is associated to addiction measures in a realistic model of AUD. Moreover, low abundance bacteria coincided with changes in central gene expression.

Converging evidence suggests that for some alcoholics (probably 30–50% of the total), ethanol consumption alters the gut microbiome by depleting protective bacteria, increasing intestinal permeability and releasing inflammation factors like bacterial peptidoglycans and lipopolysaccharide, which ultimately amplifies the psychopathology of alcoholism (de Timary et al., 2017, 2015; Gorky and Schwaber, 2016; Leclercq et al., 2014a, 2014b, 2012). However, authors suggested that alterations in microbiota composition could be responsible for the 'leaky gut' upon alcohol consumption, as no increase in permeability was observed in alcoholic patients which were resilient to microbiota changes, despite their alcohol consumption (de Timary et al., 2015). With alterations in microbial composition reported in only a subset of alcoholic patients and not correlated to the duration of sobriety, alcohol-related microbial imbalance is considered a long-lasting consequence that persists despite abstinent periods (Mutlu et al., 2012). Also, personality traits associated with the vulnerability to develop AUD have been consistently linked to gut microbial and peripheral metabolite level alterations. In particular, alcoholic patients without overt microbiota disturbances showed less severe levels of depression, anxiety and craving which almost disappeared after nearly 3 weeks of withdrawal, whereas these clinical signs persisted in abstinent patients with concurrent microbial changes (de Timary et al., 2015; Leclercq et al., 2014b, 2012). This intriguing observation poses the question of whether the gut microbiome

composition could represent a biological marker of the vulnerability to develop AUD. Intriguingly, we report here, for the first time to the best of our knowledge, that many of the biobehavioral traits associated with a loss of control-prone phenotype, without heavy ethanol intoxication, correlate with microbiome composition.

It is important to note that rats in this study underwent a 3-month period of abstinence before microbiome analyses in caecal contents and measures of mRNA in striatal areas of the brain. Therefore, measures reported here do not correlate with acute ethanol intoxication, but rather reflect long-lasting behavioral traits, i.e. the loss of control-prone phenotype observed in vulnerable rats versus the temperate behavioral profile reported in resilient animals. Our observations are in line with those reported above regarding a role for microbiota composition in negative reinforcement processes driving alcohol consumption (de Timary et al., 2017, 2015). However, our study presents two limitations that need to be addressed in the near future, 1) fecal analyses before alcohol training would inform on pre-existing compositional differences in microbiome in rats developing uncontrolled alcohol seeking behavior over time, and 2) measures of peripheral markers would inform on systemic inflammation occurring in vulnerable rats compared to resilient ones. Nevertheless, this is the first study reporting that microbiome composition is associated to addictive behavioral traits as opposed to acute effects of drug exposure.

Here we took advantage of normal variation in behavioural traits relevant to addiction to stratify an outbred cohort into either Vulnerable or Resilient. Not surprisingly, rats with impulsive traits were at higher risk of developing AUD, and this pre-existing impulsive trait shifted towards a compulsive-like behavior after extensive instrumental conditioning, associated with increased relapse rates after a period of protracted

abstinence. Of particular relevance, these animals still exhibited higher mRNA expression of D1 receptors and lower mRNA expression of D2 receptor within the dorsal striatum after a prolonged period of abstinence. The striatum is mainly composed of medium spiny neurons (MSN), typically divided into those expressing dopamine receptor D1, forming the so-called direct pathway, and those expressing D2 receptor (indirect pathway). Whereas D1-MSNs mediate reinforcement and reward, D2-MSNs have been associated with aversion and avoidance. A current consensus suggest that D1-MSNs may facilitate the selection of rewarding actions encoded in the cortex, while D2-MSNs may help to suppress cortical patterns that encode maladaptive or non-rewarding actions. Therefore, positive reinforcement learning would be modulated by signaling within the D1 direct pathway while negative reinforcement learning would be modulated by signaling within the D2 indirect pathway (Cox et al., 2015; Soares-Cunha et al., 2016; Volkow et al., 2013). As a consequence, it is postulated that dopamine-related impulsive phenotype partly relies on impaired negative feedback learning (Dagher and Robbins, 2009). Functionally, in humans, the A1 (T) allele of the dopamine D2 receptor/ankyrin repeat and kinase domain containing 1 (DRD2/ANKK1) TaqIA (rs1800497) single nucleotide polymorphism has been associated with reduced striatal D2 receptor availability (Eisenstein et al., 2016), and a recent large-scale meta-analysis confirmed the association between the ANKK1/DRD2 Taq1A polymorphism and alcoholism (Wang et al., 2013). Therefore, the lower expression of striatal D2 receptors, concomitant with higher expression of D1 receptors, in Vulnerable rats long after their last alcohol consumption confirms the construct validity of our model, and questions on the significance of those persistent brain adaptations occurring concomitantly with gut microbiota composition.

The most profound correlations were seen in D2R mRNA expression corresponding to the inhibitory, indirect pathway. Significant correlations revealed changes in low abundance genera *Lachnospiraceae UCG-006, Syntrophococcus, Shuttleworthia, Gemella, Allobaculum*, uncultured rumen bacterium from *Clostridiale vadinBB60* group, and Hydrogenoanaerobacterium associated to reductions in D2R. This novel finding indicates that gut microbiota composition may contribute to inhibitory innervations in brain circuits associated to addiction. The capability of gut microbiota to influence inhibitory circuits is not surprising given the fact that administration of *Lactobacillus rhamnosus* (JB-1) reduces anxious behaviour by altering cortical GABAergic innervations (Bravo et al., 2011).

Many genus level bacteria in order Clostridiales, family Ruminococcaceae and Lachnospiraceae, were positively associated to AUD severity, and also correlated to decreased D2R mRNA expression. Correlations between genus level bacteria and addiction measures would indicate that although these genera are not significantly different by group, subtle variations in abundance may potentially coincide with differences in addictive behaviour. While such a correlation opens a debate and requires further investigation on the mechanism linking gut microbiota to striatal D2R mRNA expression, recent evidence offers a partial explanation with the demonstration that gut microbiota regulate microRNA expression in the amygdala and prefrontal cortex. In particular, antibiotic treatment was shown to decrease miR-206-3p, a miRNA implicated in the regulation of brain-derived neurotrophic factor, essential in synaptic plasticity (Hoban et al., 2017). Rare are the studies showing that altered microbiome impacts reward seeking behaviors, but a recent study reports that microbiome-depleted animals (following antibiotic treatment) exhibited an enhanced sensitivity to cocaine

reward (Kiraly et al., 2016) . Therefore, a link most likely exists between the microbiota, the brain and the vulnerability to drug abuse. Interestingly, this finding indicates that supplementation of these low abundance bacteria may have potential for treatment in AUD, but future studies are required to investigate if probiotic/prebiotic intervention targeting the gut-brain axis (aka. psychobiotics) is capable of reducing alcohol-seeking behaviors (Dinan et al., 2013; Hoban et al., 2017).

The lack of significant differences in microbiome composition may be due to the 3-month abstinence period, however this wash-out period was chosen to ensure observed differences were not due to drug administration. Non-significant trends in altered microbiome composition between Vulnerable and Resilient group were seen in bacteria from family Ruminococcaceae and Lachnospiraceae. These findings are in line with alcohol studies showing reductions in bacteria from family Ruminococcaceae, increases in bacteria from family Lachnospiraceae, and increased alpha diversity associated to alcohol severity and altered intestinal permeability (Leclercq et al., 2014b; Llopis et al., 2016). Interestingly, it has been shown in patients with hepatic encephalopathy that the levels of Ruminococcaceae correlate negatively to inflammation (Bajaj et al., 2012). Behavioral traits, such as impulsivity, predispose individuals to addiction and other neuropsychiatric conditions, such as Attention Deficit Hyperactivity Disorder (ADHD) and autism. Previous work showed that reductions in genera *Instestimonas* (family Ruminococcaceae) and *Desulfovibrio* (family Desulfovibrionaceae) were associated to gastrointestinal dysfunction, altered metabolism, as well as anti-social, anxious, and compulsive behaviors in a mouse model of autism (Golubeva et al., 2017).

With current pharmacotherapies largely unsatisfactory, discovering novel alternatives to prevent AUD becomes a priority. Hence, identifying biological markers predicting

vulnerability to develop excessive alcohol consumption may lead to a real improvement of clinical care. In this study, we report that gut microbiome composition is associated with specific “at risk” behavioral traits in a translationally relevant model of alcohol use disorder. These preclinical observations open a debate on the possible role of gut microbiome in predisposing individuals to alcohol use disorder and offers a perspective on understanding alcohol addiction the etiology of which remains partially unknown. While addressing addiction-related associations to gut microbiome composition is probably not a panacea, it offers itself as an important underappreciated additional component in favor of better identifying those at risks of losing control over their alcohol intake.

Declaration of interest

The authors have no potential conflicts of interest to report.

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References

Bajaj, J.S., Hylemon, P.B., Ridlon, J.M., Heuman, D.M., Daita, K., White, M.B., Monteith, P., Noble, N. a., Sikaroodi, M., Gillevet, P.M., 2012. Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. AJP Gastrointest. Liver Physiol. 303, G675–G685. https://doi.org/10.1152/ajpgi.00152.2012

Barrio, P., Reynolds, J., García-Altés, A., Gual, A., Anderson, P., 2017. Social costs of illegal drugs, alcohol and tobacco in the European Union: A systematic review. Drug Alcohol Rev. 36, 578–588. https://doi.org/10.1111/dar.12504

Baumberg, B., 2006. The global economic burden of alcohol: A review and some suggestions. Drug Alcohol Rev. 25, 537–551. https://doi.org/10.1080/09595230600944479

Bravo, J. a., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J., Cryan, J.F., 2011. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc. Natl. Acad. Sci. 108, 16050–16055. https://doi.org/10.1073/pnas.1102999108

Bull-Otterson, L., Feng, W., Kirpich, I., Wang, Y., Qin, X., Liu, Y., Gobejishvili, L., Joshi-Barve, S., Ayvaz, T., Petrosino, J., Kong, M., Barker, D., McClain, C., Barve, S., 2013. Metagenomic Analyses of Alcohol Induced Pathogenic Alterations in the Intestinal Microbiome and the Effect of Lactobacillus rhamnosus GG Treatment. PLoS One 8, 4–13. https://doi.org/10.1371/journal.pone.0053028

Clarke, G., Stilling, R.M., Kennedy, P.J., Stanton, C., Cryan, J.F., Dinan, T.G., 2014. Minireview: Gut Microbiota: The Neglected Endocrine Organ. Mol. Endocrinol. 28, 1221–1238. https://doi.org/10.1210/me.2014-1108

Collins, S.M., Kassam, Z., Bercik, P., 2013. The adoptive transfer of behavioral phenotype via the intestinal microbiota: Experimental evidence and clinical implications. Curr. Opin. Microbiol. 16, 240–245. https://doi.org/10.1016/j.mib.2013.06.004

Cox, S.M.L., Frank, M.J., Larcher, K., Fellows, L.K., Clark, C. a., Leyton, M., Dagher, A., 2015. Striatal D1 and D2 signaling differentially predict learning from positive and negative outcomes. Neuroimage 109, 95–101. https://doi.org/10.1016/j.neuroimage.2014.12.070

Crews, F.T., Lawrimore, C.J., Walter, T.J., Coleman, L.G., 2017a. The role of neuroimmune signaling in alcoholism. Neuropharmacology 122, 56–73. https://doi.org/10.1016/j.neuropharm.2017.01.031

Crews, F.T., Qin, L., Sheedy, D., Vetreno, R.P., Zou, J., 2013. High mobility group box 1/toll-like receptor danger signaling increases brain neuroimmune activation in alcohol dependence. Biol. Psychiatry 73, 602–612. https://doi.org/10.1016/j.biopsych.2012.09.030

Crews, F.T., Walter, T.J., Coleman, L.G., Vetreno, R.P., 2017b. Toll-like receptor signaling and stages of addiction. Psychopharmacology (Berl). 234, 1483–1498. https://doi.org/10.1007/s00213-017-4560-6

Cryan, J.F., Dinan, T.G., 2012. Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. Nat. Rev. Neurosci. 13, 701–712. https://doi.org/10.1038/nrn3346

Dagher, A., Robbins, T.W., 2009. Personality, Addiction, Dopamine: Insights from Parkinson's Disease. Neuron 61, 502–510. https://doi.org/10.1016/j.neuron.2009.01.031

De Timary, P., Leclercq, S., Stärkel, P., Delzenne, N., 2015. A dysbiotic subpopulation of alcohol-dependent subjects. Gut Microbes 6, 388–391. https://doi.org/10.1080/19490976.2015.1107696

De Timary, P., Stärkel, P., Delzenne, N.M., Leclercq, S., 2017. A role for the peripheral immune system in the development of alcohol use disorders? Neuropharmacology 122, 148–160. https://doi.org/10.1016/j.neuropharm.2017.04.013

Deroche-gamonet, V., Belin, D., Piazza, P.V., 2004. Evidence for Addiction-lîke Behavior in the Rat. Science (80-.). 1014, 1014–1018. https://doi.org/10.1126/science.1099020

Dinan, T.G., Cryan, J.F., 2017. The Microbiome-Gut-Brain Axis in Health and Disease. Gastroenterol. Clin. North Am. 46, 77–89. https://doi.org/10.1016/j.gtc.2016.09.007

Dinan, T.G., Stanton, C., Cryan, J.F., 2013. Psychobiotics: A novel class of psychotropic. Biol. Psychiatry 74, 720–726. https://doi.org/10.1016/j.biopsych.2013.05.001

Dinan, T.G., Stilling, R.M., Stanton, C., Cryan, J.F., 2015. Collective unconscious: How gut microbes shape human behavior. J. Psychiatr. Res. 63, 1–9. https://doi.org/10.1016/j.jpsychires.2015.02.021

Doherty, F.D., O'Mahony, S.M., Peterson, V.L., O'Sullivan, O., Crispie, F., Cotter, P.D., Wigmore, P., King, M. V., Cryan, J.F., Fone, K.C.F., 2017. Post-weaning social isolation of rats leads to long-term disruption of the gut microbiota-immune-brain axis. Brain. Behav. Immun. 68, 261–273. https://doi.org/10.1016/j.bbi.2017.10.024

Eisenstein, S. a., Bogdan, R., Love-Gregory, L., Corral-Frías, N.S., Koller, J.M., Black, K.J., Moerlein, S.M., Perlmutter, J.S., Barch, D.M., Hershey, T., 2016. Prediction of striatal D2 receptor binding by DRD2/ANKK1 TaqIA allele status. Synapse 70, 418–431. https://doi.org/10.1002/syn.21916

Golubeva, A. V., Joyce, S. a., Moloney, G., Burokas, A., Sherwin, E., Arboleya, S., Flynn, I., Khochanskiy, D., Moya-Pérez, A., Peterson, V., Rea, K., Murphy, K., Makarova, O., Buravkov, S., Hyland, N.P., Stanton, C., Clarke, G., Gahan, C.G.M., Dinan, T.G., Cryan, J.F., 2017. Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism. EBioMedicine 24, 166–178. https://doi.org/10.1016/j.ebiom.2017.09.020

Gorky, J., Schwaber, J., 2016. The role of the gut-brain axis in alcohol use disorders. Prog. Neuro-Psychopharmacology Biol. Psychiatry 65, 234–241. https://doi.org/10.1016/j.pnpbp.2015.06.013

Hoban, A.E., Stilling, R.M., M. Moloney, G., Moloney, R.D., Shanahan, F., Dinan, T.G., Cryan, J.F., Clarke, G., 2017. Microbial regulation of microRNA expression in the amygdala and prefrontal cortex. Microbiome 5, 102. https://doi.org/10.1186/s40168-017-0321-3

Jadhav, K.S., Magistretti, P.J., Halfon, O., Augsburger, M., Boutrel, B., 2017. A preclinical model for identifying rats at risk of alcohol use disorder. Sci. Rep. 7, 9454. https://doi.org/10.1038/s41598-017-09801-1

Kiraly, D.D., Walker, D.M., Calipari, E.S., Labonte, B., Issler, O., Pena, C.J., Ribeiro, E. a., Russo, S.J., Nestler, E.J., 2016. Alterations of the host microbiome affect behavioral responses to cocaine. Sci. Rep. 6, 1–12. https://doi.org/10.1038/srep35455

Leclercq, S., Cani, P.D., Neyrinck, A.M., Stärkel, P., Jamar, F., Mikolajczak, M., Delzenne, N.M., De Timary, P., 2012. Role of intestinal permeability and inflammation in the biological and behavioral control of alcohol-dependent subjects. Brain. Behav. Immun. 26, 911–918. https://doi.org/10.1016/j.bbi.2012.04.001

Leclercq, S., De Saeger, C., Delzenne, N., De Timary, P., Stärkel, P., 2014a. Role of inflammatory pathways, blood mononuclear cells, and gut-derived bacterial products in alcohol dependenc. Biol. Psychiatry 76, 725–733. https://doi.org/10.1016/j.biopsych.2014.02.003

Leclercq, S., De Timary, P., Delzenne, N.M., Stärkel, P., 2017. The link between inflammation, bugs, the intestine and the brain in alcohol dependence. Transl. Psychiatry 7. https://doi.org/10.1038/tp.2017.15

Leclercq, S., Matamoros, S., Cani, P.D., Neyrinck, A.M., Jamar, F., Stärkel, P., Windey, K., Tremaroli, V., Bäckhed, F., Verbeke, K., de Timary, P., Delzenne, N.M., 2014b. Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. Proc. Natl. Acad. Sci. 111, E4485–E4493. https://doi.org/10.1073/pnas.1415174111

Llopis, M., Cassard, a. M., Wrzosek, L., Boschat, L., Bruneau, a., Ferrere, G., Puchois, V., Martin, J.C., Lepage, P., Le Roy, T., Lefèvre, L., Langelier, B., Cailleux, F., González-Castro, a. M., Rabot, S., Gaudin, F., Agostini, H., Prévot, S., Berrebi, D., Ciocan, D., Jousse, C., Naveau, S., Gérard, P., Perlemuter, G., 2016. Intestinal microbiota contributes to individual susceptibility to alcoholic liver disease. Gut 65, 830–839. https://doi.org/10.1136/gutjnl-2015-310585

Mann, K., Hermann, D., 2010. Individualised treatment in alcohol-dependent patients. Eur. Arch. Psychiatry Clin. Neurosci. 260, 116–120. https://doi.org/10.1007/s00406-010-0153-7

McVey Neufeld, K.A., Luczynski, P., Dinan, T.G., Cryan, J.F., 2016. Reframing the teenage wasteland: Adolescent microbiota-gut-brain axis. Can. J. Psychiatry 61, 214–221. https://doi.org/10.1177/0706743716635536

Montiel-Castro, A.J., González-Cervantes, R.M., Bravo-Ruiseco, G., Pacheco-López, G., 2013. The microbiota-gut-brain axis: neurobehavioral correlates, health and sociality. Front. Integr. Neurosci. 7, 1–16. https://doi.org/10.3389/fnint.2013.00070

Mutlu, E. a., Gillevet, P.M., Rangwala, H., Sikaroodi, M., Naqvi, a., Engen, P. a., Kwasny, M., Lau, C.K., Keshavarzian, a., 2012. Colonic microbiome is altered in alcoholism. AJP Gastrointest. Liver Physiol. 302, G966–G978. https://doi.org/10.1152/ajpgi.00380.2011

Peterson, V.L., Jury, N.J., Cabrera-Rubio, R., Draper, L. a., Crispie, F., Cotter, P.D., Dinan, T.G., Holmes, A., Cryan, J.F., 2017. Drunk bugs: Chronic vapour alcohol exposure induces marked changes in the gut microbiome in mice. Behav. Brain Res. 323, 172–176. https://doi.org/10.1016/j.bbr.2017.01.049

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Doré, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P., Ehrlich, S.D., Wang, J., Antolin, M., Artiguenave, F., Blottiere, H., Borruel, N., Bruls, T., Casellas, F., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Forte, M., Friss, C., Van De Guchte, M., Guedon, E., Haimet, F., Jamet, A., Juste, C., Kaci, G., Kleerebezem, M., Knol, J., Kristensen, M., Layec, S., Le Roux, K., Leclerc, M., Maguin, E., Melo Minardi, R., Oozeer, R., Rescigno, M., Sanchez, N., Tims, S., Torrejon, T., Varela, E., De Vos, W., Winogradsky, Y., Zoetendal, E., 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65. https://doi.org/10.1038/nature08821

Rea, K., Dinan, T.G., Cryan, J.F., 2016. The microbiome: A key regulator of stress and neuroinflammation. Neurobiol. Stress 4, 23–33. https://doi.org/10.1016/j.ynstr.2016.03.001

Sacks, J.J., Gonzales, K.R., Bouchery, E.E., Tomedi, L.E., Brewer, R.D., 2015. 2010 National and State Costs of Excessive Alcohol Consumption. Am. J. Prev. Med. 49, e73–e79. https://doi.org/10.1016/j.amepre.2015.05.031

Saitz, R., 2009. Unhealthy Alcohol Use. http://dx.doi.org.gate2.inist.fr/10.1056/NEJMcp042262 15–17.

Sampson, T.R., Mazmanian, S.K., 2015. Control of brain development, function, and behavior by the microbiome. Cell Host Microbe 17, 565–576. https://doi.org/10.1016/j.chom.2015.04.011

Sherwin, E., Dinan, T.G., Cryan, J.F., 2017. Recent developments in understanding the role of the gut microbiota in brain health and disease. Ann. N. Y. Acad. Sci. 17, 1–21. https://doi.org/10.1111/nyas.13416

Soares-Cunha, C., Coimbra, B., Sousa, N., Rodrigues, A.J., 2016. Reappraising striatal D1- and D2-neurons in reward and aversion. Neurosci. Biobehav. Rev. 68, 370–386. https://doi.org/10.1016/j.neubiorev.2016.05.021

Temko, J.E., Bouhlal, S., Farokhnia, M., Lee, M.R., Cryan, J.F., Leggio, L., 2017. The microbiota, the gut and the brain in eating and alcohol use disorders: A “ménage à trois”? Alcohol Alcohol. 52, 403–413. https://doi.org/10.1093/alcalc/agx024

Volkow, N.D., Wang, G.J., Tomasi, D., Baler, R.D., 2013. Unbalanced neuronal circuits in addiction. Curr. Opin. Neurobiol. 23, 639–648. https://doi.org/10.1016/j.conb.2013.01.002

Wang, F., Simen, A., Arias, A., Lu, Q.W., Zhang, H., 2013. A large-scale meta-analysis of the association between the ANKK1/DRD2 Taq1A polymorphism and alcohol dependence. Hum. Genet. 132, 347–358. https://doi.org/10.1007/s00439-012-1251-6

World Health Organization. Global status report on alcohol and health. 2014

Legends to figures

Figure 1: Identification of rats at risk of alcohol use disorder. **(A)** Flowchart of the experimental procedure. **(B)** Distribution of the 59 rats: 26 rats in the 0 criterion group, 14 rats in the 1 criterion group, 12 rats in the 2 criteria group, 7 rats in the 3 criteria group. **(C)** Persistence in lever pressing during no-drug period: One-way ANOVA showed a significant difference ($F_{3,55} = 17.436$, $p < 0.0001$). Post hoc Bonferroni's test revealed that the 3crit rats exhibited higher lever presses compared to the 0Crit ($p < 0.0001$), the 1Crit $p=0.0048$) but not as compared to the 2Crit rats ($p=0.96$). The 2Crit rats differed from 0Crit rats ($p < 0.001$) and 1Crit rats ($p = 0.0012$). The 0 and 1 criterion rats had similar performances ($p = 0.149$). * Significant compared to 0Crit and 1Crit rats. **(D)** Motivation on progressive ratio: One-way ANOVA showed a statistically significant difference ($F_{3,55} = 23.23$, $p < 0.0001$). Post hoc Bonferroni's tests revealed that the 3Crit rats displayed an increased motivation for ethanol seeking compared to 0crit rats ($p < 0.0001$), and 1Crit rats ($p=0.0018$) but not as compared to the 2Crit rats ($p=0.62$). The 2Crit rats displayed a higher breaking point compared to 0Crit ($p < 0.001$) and 1Crit rats ($p = 0.002$). The 1crit rats exhibited a higher breaking point compared to the 0Crit rats ($p = 0.001$). * Significant compared to 0Crit and 1Crit rats. # Significant compared to 0Crit rats. **(E)** Alcohol seeking in presence of shock: One-way ANOVA showed a statistically significant difference ($F_{3,55} = 22.55$, $p < 0.0001$). Post hoc Bonferroni's tests revealed that 3crit rats accepted more shocks than 2Crit (<0.0001), 1Crit ($p < 0.0001$) and 0Crit rats ($p = 0.002$). Whereas 2Crit rats were not different from 1Crit rats ($p = 0.372$), they exhibited a higher resistance to punishment as compared to the 0Crit rats ($p = 0.002$). Finally, 1Crit rats had higher lever presses than 0Crit rats ($p=0.0002$). # Significant compared to 0Crit rats. @ Significant compared to 0Crit, 1Crit and 2Crit rats. **(F)** Addiction Score: A one-way ANOVA revealed a significant difference ($F_{3,55} = 67.20$, $p<0.0001$). A post hoc Bonferroni's test showed the each group was significantly different from the other groups. This shows that the addiction score is highly representative of the three criteria scores. #Significant compared to 0Crit rats. *Significant compared to 0Crit and 1Crit rats. @Significant compared to 0Crit, 1Crit and 2Crit rats. **(G)** Reinstatement: A one-way ANOVA revealed a significant difference

between the groups ($F_{3,55}$ = 6.13, p=0.0012). A post hoc Bonferroni's test showed that the 2Crit and 3Crit rats had higher active lever presses as compared to 0Crit (p<0.01) and 1Crit (p<0.001). We chose to group 0Crit and 1Crit rats, labelled them as Resilient and grouped 2Crit and 3Crit, and labelled them as Vulnerable. * Significant compared to 0Crit and 1Crit rats. **(H)** Partial grid paradigm: A two-way repeated measures ANOVA with repetition in one factor identified a significant group effect ($F_{1,56}$ = 16.62, p<0.0001), a significant effect of intensity of the shock ($F_{2,116}$ = 105.89, p<0.001) and but no interaction effect ($F_{112,173}$ = 2.07, p=0.1298). **(I)** Effect of Baclofen on progressive ratio: A two-way repeated measures ANOVA with repetition in one factor identified a significant group effect ($F_{1,56}$ = 11.09, p=0.0015), a significant dose effect ($F_{2,116}$ = 25.71, p<0.0001) and a significant interaction effect ($F_{112,173}$ = 10.46, p<0.0001). A group-wise post hoc Bonferroni's test showed that both the doses reduced the motivation for ethanol intake in the Vulnerable group, while only the 2 mg/kg dose had an effect in the Resilient group of rats. ^ Significant compared to 0 mg/kg in Vulnerable rats. & Significant compared to 0mg/kg in respective groups. **(J)** Effect of Baclofen on Reinstatement: A two-way repeated measures ANOVA with repetition in one factor identified a significant group effect ($F_{1,55}$ = 9.25, p=0.0036), a significant dose effect ($F_{1,55}$ = 67.81, p<0.0001) and a significant interaction effect ($F_{55,113}$= 7.55, p=0.008). This indicates that baclofen had a more pronounced effect in the Vulnerable group of rats as compared to Resilient rats. **(K)** Impulsivity on 5CSRTT: The average percentage of premature responses was 40.34 ± 1.64 and 47.37 ± 2.7, for Resilient and Vulnerable rats, respectively. An Unpaired T-test (t57 = -2.315, p=0.02) revealed that the Vulnerable group had a significantly higher number of premature responses as compared to the Resilient group. *Significant compared to Resilient rats. **(L)** Factor Analysis: All the five variables loaded on one construct (Persistence in drug seeking during the no-drug period: r = 0.719, Excessive motivation for alcohol seeking: r = 0.832, Resistance to punishment: r = 0.715, reinstatement: r = 0.658 and impulsivity: r=0.65) accounting for 50% of the variance, further supporting that the three addiction-like criteria, reinstatement and the pre-existing trait of impulsivity are measures of a single underlying factor.

Figure 2: Selection of most Resilient (grey, n=19, combining 18 0Crit and 1 1Crit rats) and most Vulnerable (red, n=19, combining twelve 2Crit and seven 3Crit rats) animals among the 59 rats screened, according to (A) Persistence in lever pressing during no-drug period, (B) Motivation on progressive ratio schedule, (C) Alcohol seeking in presence of shock, (D) Addiction Score, (E) Reinstatement and (F) Response to baclofen assessed in a progressive ratio schedule. Significance codes: *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

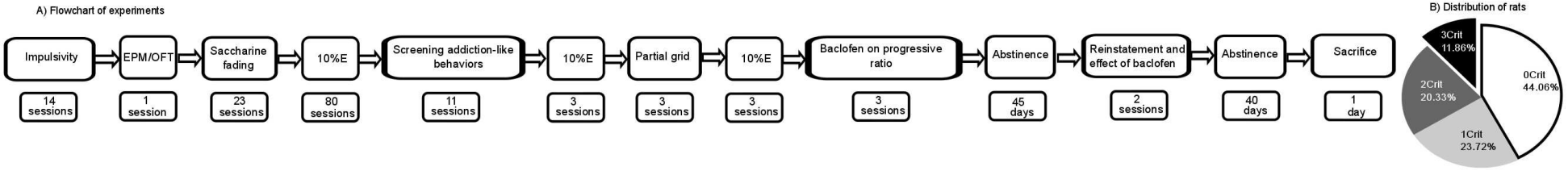
Figure 3: A posteriori analyses revealed that Vulnerable rats exhibited increased impulsivity (A), similar exploration in an elevated plus maze (B), increased novelty induced locomotion (C), similar alcohol intake after prolonged conditioning (D), increased D1R mRNA (E) and decreased D2R mRNA expression (F) in the dorsal striatum compared to Resilient rats. In bar graphs all samples are plotted as grey dots. In box-and-whisker plots outliers are indicated with a black dot. Resilient group (grey) is plotted on left, Vulnerable group (red) on right. Significance codes: *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

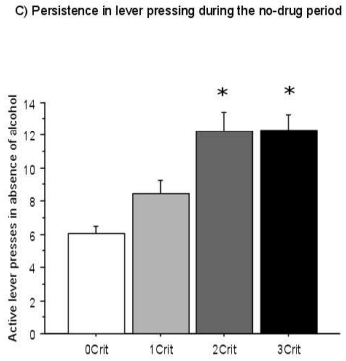
Figure 4: Microbiome Diversity. A) Alpha diversity index measures of bacterial richness (chao1 index and observed_species index). B) Alpha diversity index measure of bacterial richness and evenness (shannon index). In box-and-whisker plots outliers are indicated with a black dot. C) PCoA ordination plot of orthogonal taxonomic unit (OUT) beta diversity. Percent explained variance reported on first (x-axis) and second (y-axis) principal component axis. Density of cluster indicated for the y-axis (right) and x-axis (bottom) by group.

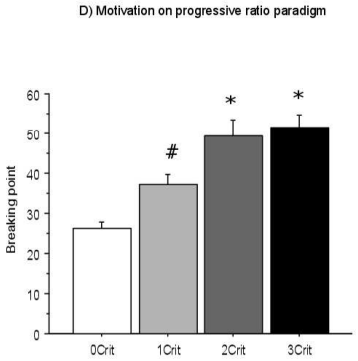
Figure 5: Genus Level Relative Abundance. The mean of the 22 most abundant bacteria are plotted by group, with the inner ring representing Vulnerable and outer ring representing Resilient group. Legend displays bacterial name with taxonomic

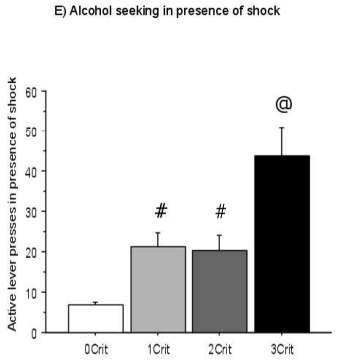
designation at each level down to genus; bacteria are listed by order of appearance starting with *Bacteroides* (blue) at top.

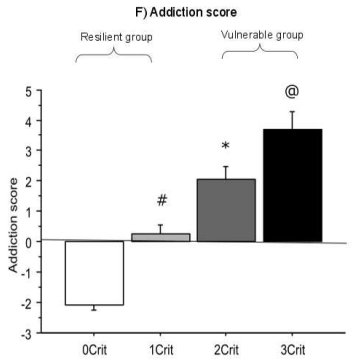
Figure 6: Left panel - Correlations (positive in red, negative in blue) between genus-level bacteria (y-axis) and addiction measures (x-axis); Right panel – Log2 fold change ratio showing changes in Vulnerable group relative to Resilient (increases in red, decreases in blue) for genera corresponding to correlation heatmap (left panel). Abbreviations (left to right x-axis): active lever presses without ethanol (LP.woEtOH), active lever presses with shock (LP.wShock), progressive ratio breaking point (PR), addiction score (Addiction.score), criteria designation 0Crit-3Crit (Criteria), percentage of premature responses in 5 choice serial reaction time task (5CSRTT), percent time in open arm in elevate plus maze (EPM), locomotor activity in open field test (OFT), total ethanol consumed over 80 sessions (TotEtOH), dopamine 1 receptor mRNA expression (D1R), dopamine 2 receptor mRNA expression (D2R), change in progressive ratio breaking point between 0mg/kg dosage and 1mg/kg dosage baclofen (dPR.baclofen), active lever presses during reinstatement (Reinstatement).

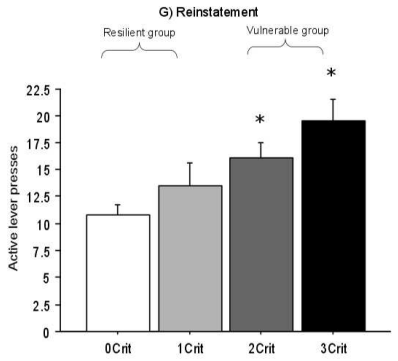

A) Flowchart of experiments
Impulsivity
14 sessions
EPM/OFT
1 session
Saccharine fading
23 sessions
10%E
80 sessions
Screening addiction-like behaviors
11 sessions
10%E
3 sessions
Partial grid
3 sessions
10%E
3 sessions
Baclofen on progressive ratio
3 sessions
Abstinence
45 days
Reinstatement and effect of baclofen
2 sessions
Abstinence
40 days
Sacrifice
1 day
B) Distribution of rats
3Crit 11.86%
0Crit 44.06%
2Crit 20.33%
1Crit 23.72%

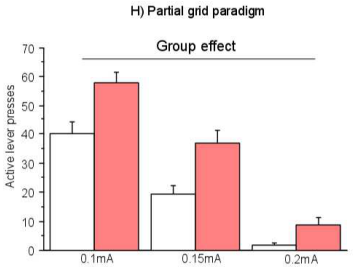

C) Persistence in lever pressing during the no-drug period
Active lever presses in absence of alcohol
0Crit
1Crit
2Crit
3Crit

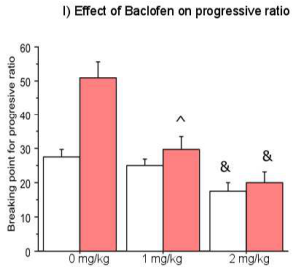

D) Motivation on progressive ratio paradigm
Breaking point
0Crit
1Crit
2Crit
3Crit

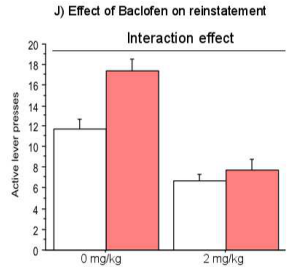

E) Alcohol seeking in presence of shock
Active lever presses in presence of shock
0Crit
1Crit
2Crit
3Crit

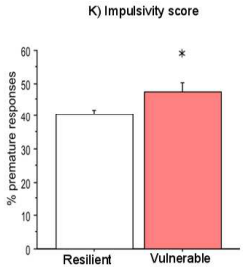

F) Addiction score
Resilient group
Vulnerable group
Addiction score
0Crit
1Crit
2Crit
3Crit

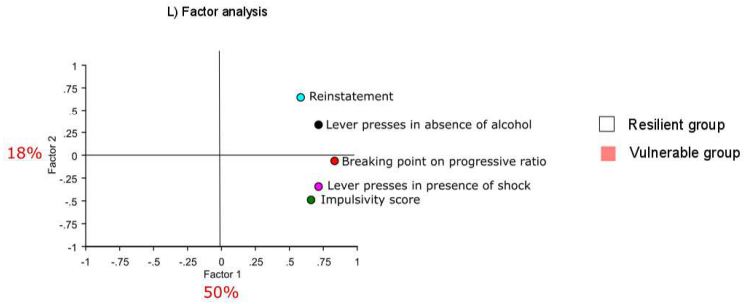

G) Reinstatement
Resilient group
Vulnerable group
Active lever presses
0Crit
1Crit
2Crit
3Crit

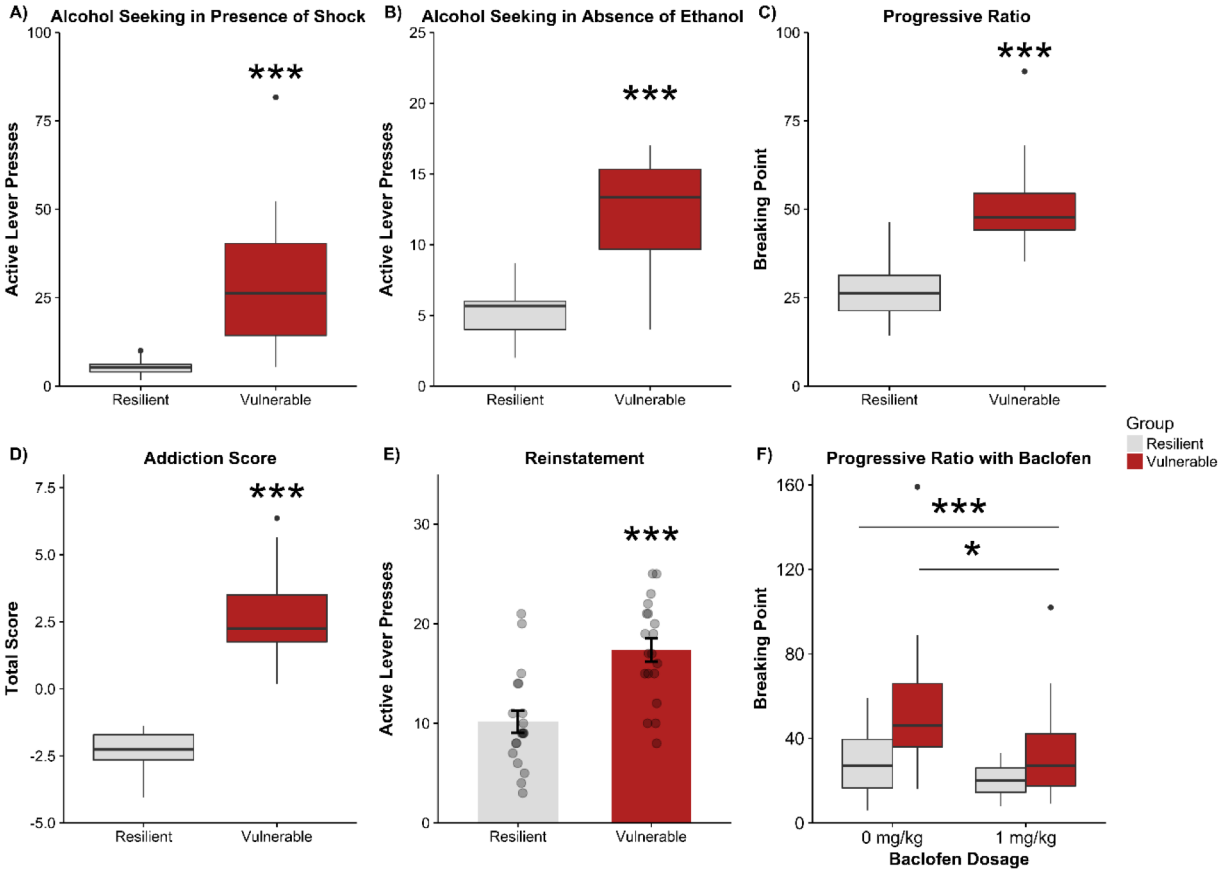

H) Partial grid paradigm
Group effect
Active lever presses
0.1mA
0.15mA
0.2mA

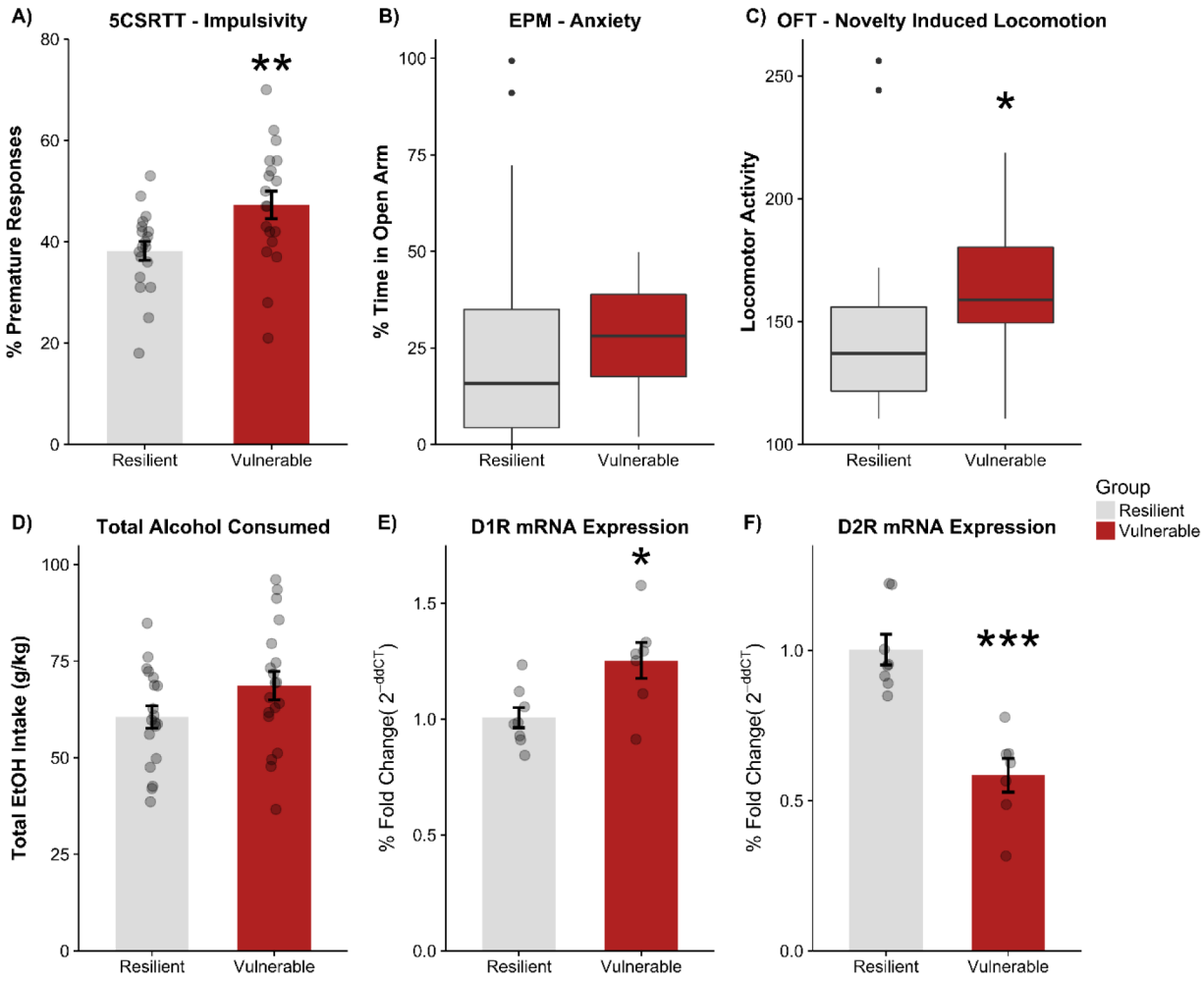

I) Effect of Baclofen on progressive ratio
Breaking point for progresive ratio
0 mg/kg
1 mg/kg
2 mg/kg

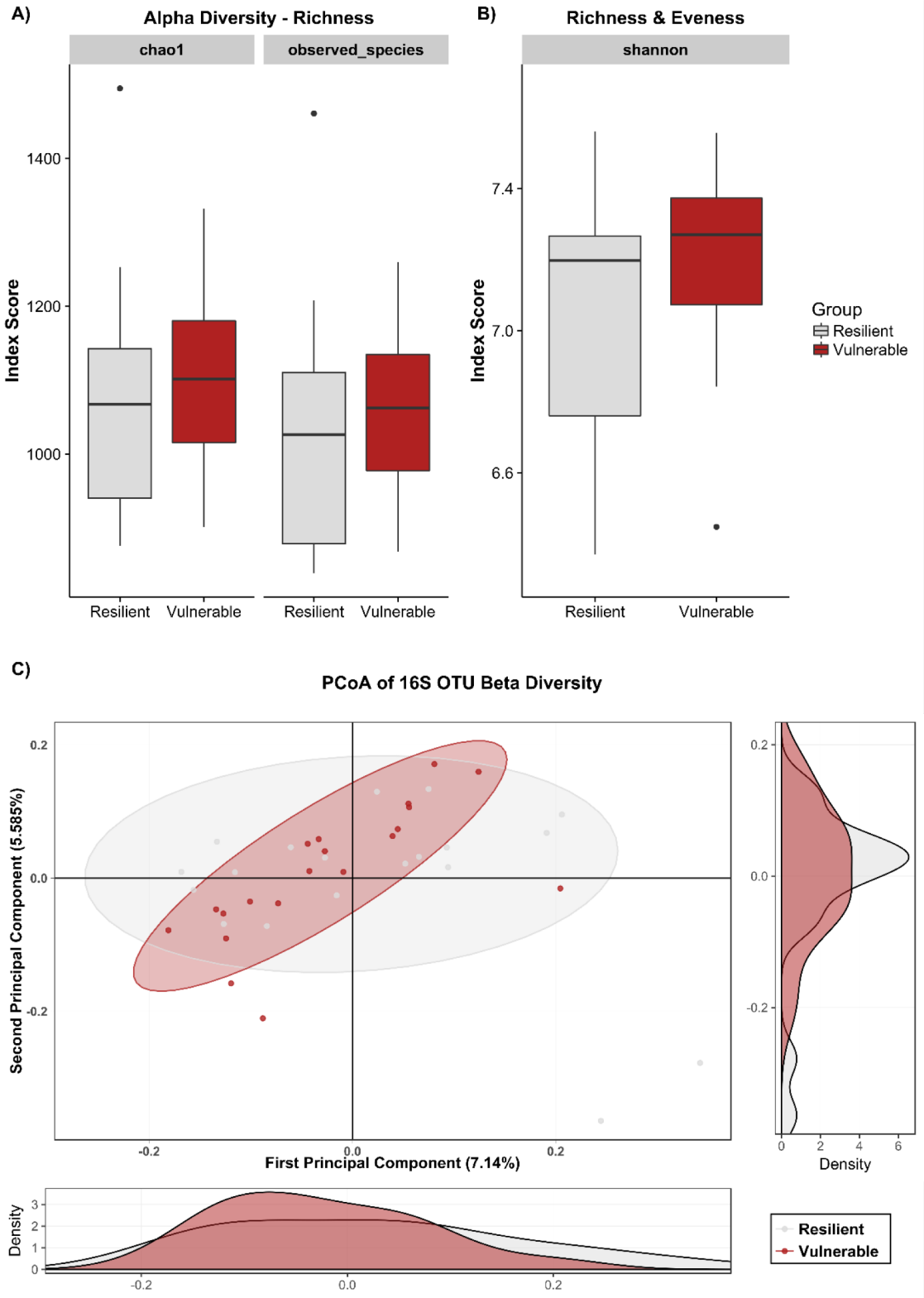

J) Effect of Baclofen on reinstatement
Interaction effect
Active lever presses
0 mg/kg
2 mg/kg


K) Impulsivity score
% premature responses
Resilient
Vulnerable


L) Factor analysis
Reinstatement
Lever presses in absence of alcohol
Breaking point on progressive ratio
Lever presses in presence of shock
Impulsivity score
18%
Factor 2
Factor 1
50%
Resilient group
Vulnerable group

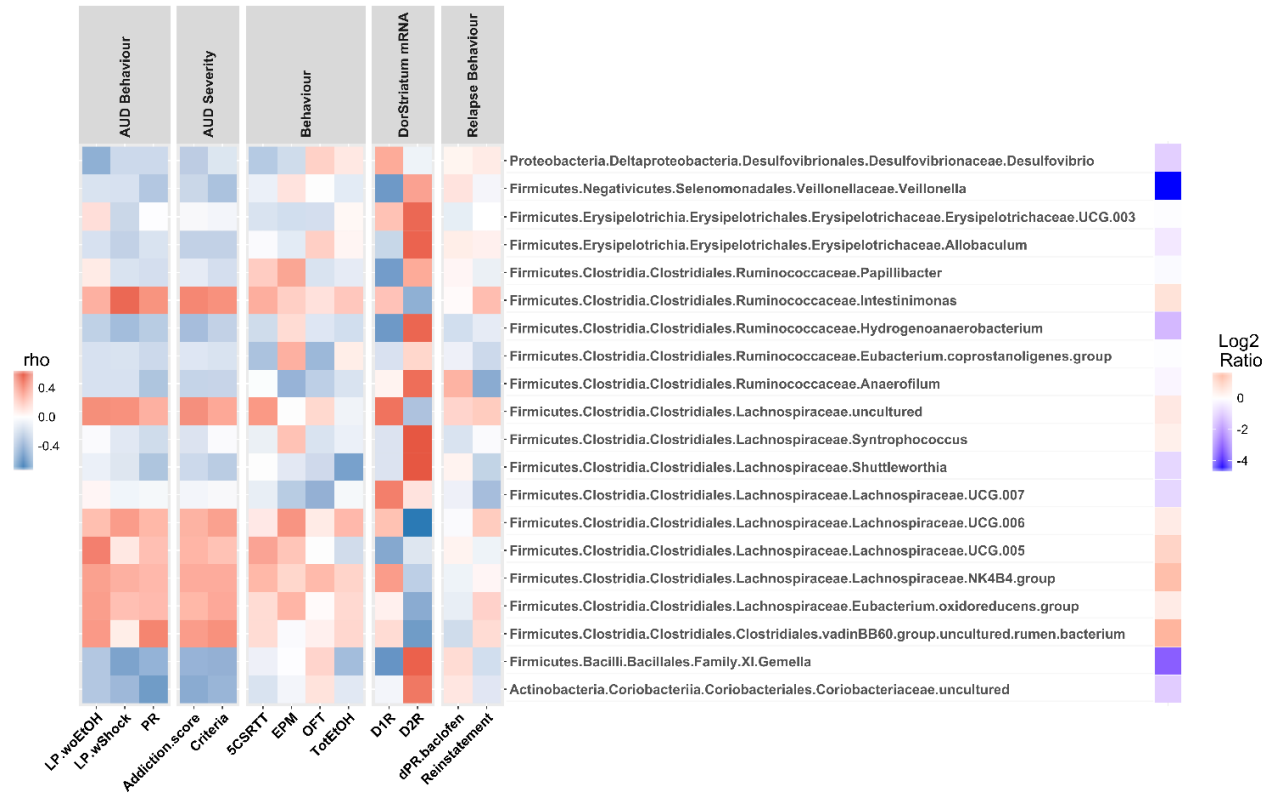

A)
Alcohol Seeking in Presence of Shock
Active Lever Presses
100
75
50
25
0
***
Resilient
Vulnerable
B)
Alcohol Seeking in Absence of Ethanol
Active Lever Presses
25
20
15
10
5
0
***
Resilient
Vulnerable
C)
Progressive Ratio
Breaking Point
100
75
50
25
0
***
Resilient
Vulnerable
Group
Resilient
Vulnerable
D)
Addiction Score
Total Score
7.5
5.0
2.5
0.0
-2.5
-5.0
***
Resilient
Vulnerable
E)
Reinstatement
Active Lever Presses
30
20
10
0
***
Resilient
Vulnerable
F)
Progressive Ratio with Baclofen
Breaking Point
160
120
80
40
0
***
*
0 mg/kg
1 mg/kg
Baclofen Dosage


A)
5CSRTT - Impulsivity
% Premature Responses
80
60
40
20
0
**
Resilient
Vulnerable
B)
EPM - Anxiety
% Time in Open Arm
100
75
50
25
0
Resilient
Vulnerable
C)
OFT - Novelty Induced Locomotion
Locomotor Activity
250
200
150
100
*
Resilient
Vulnerable
Group
Resilient
Vulnerable
D)
Total Alcohol Consumed
Total EtOH Intake (g/kg)
100
75
50
25
0
Resilient
Vulnerable
E)
D1R mRNA Expression
% Fold Change(2−ddCT)
1.5
1.0
0.5
0.0
*
Resilient
Vulnerable
F)
D2R mRNA Expression
% Fold Change(2−ddCT)
1.0
0.5
0.0
***
Resilient
Vulnerable


A)
Alpha Diversity - Richness
chao1
observed_species
Index Score
1400
1200
1000
Resilient
Vulnerable
Resilient
Vulnerable
B)
Richness & Eveness
shannon
Index Score
7.4
7.0
6.6
Resilient
Vulnerable
Group
Resilient
Vulnerable
C)
PCoA of 16S OTU Beta Diversity
Second Principal Component (5.585%)
0.2
0.0
-0.2
-0.2
0.0
0.2
First Principal Component (7.14%)
0.2
0.0
-0.2
0
2
4
6
Density
Density
3
2
1
0
-0.2
0.0
0.2
Resilient
Vulnerable

Genus Level Abundance

- Bacteroidetes.Bacteroidia.Bacteroidales.Bacteroidaceae.Bacteroides
- Bacteroidetes.Bacteroidia.Bacteroidales.Bacteroidales S24-7 group.uncultured bacterium
- Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Alloprevotella
- Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae NK3B31 group
- Cyanobacteria.Melainabacteria.Gastranaerophilales.uncultured bacterium.
- Firmicutes.Bacilli.Lactobacillales.Lactobacillaceae.Lactobacillus
- Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnoclostridium
- Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae NK4A136 group
- Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.uncultured
- Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.[Eubacterium] coprostanoligenes group
- Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Oscillibacter
- Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminiclostridium 5
- Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminiclostridium 6
- Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminiclostridium 9
- Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcaceae UCG-013
- Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcaceae UCG-014
- Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcus 1
- Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.uncultured
- Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrio
- Proteobacteria.Epsilonproteobacteria.Campylobacterales.Helicobacteraceae.Helicobacter
- Saccharibacteria.Unknown Class.Unknown Order.Unknown Family.Candidatus Saccharimonas
- Verrucomicrobia.Verrucomicrobiae.Verrucomicrobiales.Verrucomicrobiaceae.Akkermansia
- Other


AUD Behaviour
AUD Severity
Behaviour
DorStriatum mRNA
Relapse Behaviour
Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrio
Firmicutes.Negativicutes.Selenomonadales.Veillonellaceae.Veillonella
Firmicutes.Erysipelotrichia.Erysipelotrichales.Erysipelotrichaceae.Erysipelotrichaceae.UCG.003
Firmicutes.Erysipelotrichia.Erysipelotrichales.Erysipelotrichaceae.Allobaculum
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Papillibacter
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Intestinimonas
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Hydrogenoanaerobacterium
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Eubacterium.coprostanoligenes.group
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Anaerofilum
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.uncultured
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Syntrophococcus
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Shuttleworthia
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.007
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.006
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.005
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.NK4B4.group
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Eubacterium.oxidoreducens.group
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.rumen.bacterium
Firmicutes.Bacilli.Bacillales.Family.XI.Gemella
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.uncultured
rho
0.4
0.0
-0.4
Log2 Ratio
0
-2
-4
LP.woEtOH
LP.wShock
PR
Addiction.score
Criteria
5CSRTT
EPM
OFT
TotEtOH
D1R
D2R
dPR.baclofen
Reinstatement

Supplementary information for

Gut microbiome correlates with altered striatal dopamine receptor expression in a model of compulsive alcohol seeking

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Material and methods

Apparatus:

A) Self-administration (SA) chambers

Twelve operant chambers (305 x 241 x 210 mm, Med Associates, St. Albans, Vermont, USA) were used for the experiment. The chambers were housed in larger sound attenuated cubicle, equipped with exhaust fans for air renewal, also used for masking the background noise. The floor was made of a grid capable of delivering electrical shock. Each operant panel contained two retractable levers 60 mm above the grid and 35 mm equidistant from the midline, with a white light diode mounted 30 mm above each lever. Between the two levers was the delivery section which delivered 0.1mL of the fluid by means of a dipper.

B) Chambers for testing impulsivity

Six operant chambers (305 x 241 x 292 mm) were used for the 5-Choice Serial Reaction Time Task experiment (Med Associates Inc., St-Albans, Vermont, USA). Each chamber was enclosed in wooden cubicles equipped with an exhaust fan for ventilation. Each cage contained a stainless-steel grid floor spaced by 18mm, allowing waste collection in a removable tray containing sawdust. Front and back wall of the cage were in Plexiglas, while left and right wall were made of steel. Five nosepoke cavities (25x25 mm) were located on the left side of the cage, each spaced by 25mm. A food tray located 20 mm above the grid was available on the right side. Nosepoke cavities and food tray were equipped with a light and an infrared beam to monitor activity. Each cage was also equipped with a house light fixed on the ceiling, and a tone device. All the

operant cages were linked to a common interface, and to a computer that controlled experimental procedures through Med Associates software (Med-PC IV).

Impulsivity- 5 Choice Serial Reaction Time Task (5-CSRTT).

Before the rats were exposed to alcohol, their impulsivity profile was ascertained by testing them on the 5-CSRTT. Rats were food restricted and maintained at 90% of their initial weight. For the whole experiment, sucrose pellets (Dustless precision pellet 45 mg, rodent purified diet, BioServ, Frenchtown, NJ, USA) were given as a "reward" for correct responses.

Rats were first trained to nose poke in the food tray to get a food pellet reward. Each session stopped when rats collected 50 pellets of after 30 min.

Then, on each trial, rats were trained to nose poke first in the food tray to start a trial and later nose poke in one of the five holes, randomly illuminated in order to get a food pellet reward (recorded as a correct response). Nose poking in a different hole was recorded but had no consequence. Each session stopped when rats collected 40 pellets of after 30 min.

The next training phase was similar to the previous ones, but a 5-sec delay preceded the random illumination and nose poking in a non-illuminated hole was recorded as an incorrect response, triggering a 5-sec time out period signalled by the illumination of the house light. Each session stopped when rats collected 50 pellets of after 30 min.

Then, a 5-second tone was introduced to indicate the 5-sec delay before the random illumination. At the end of the tone, one of the 5 holes was briefly illuminated for 2 seconds. Any response during the tone, i.e. before the holes being illuminated, was

recorded as a premature response but had no consequence. Correct and incorrect responses were recorded as previously explained. Absence of response during the 5 sec post-illumination was recorded as an omission.
Ultimately, rats were tested in the 5-CSRTT paradigm during which each premature response was punished by a time out of 5 seconds and the illumination of the house light. The correct, incorrect and omission responses were recorded as previously described.

Premature responses were calculated as [premature responses * 100/ (correct + incorrect + omission + premature responses)].

Anxiety-related behaviors

Rats were tested in an Open Field and in an Elevated plus maze paradigm for evaluating anxiety-like behaviors after the 5-CSRTT procedure. Both experiments were conducted under a dim light (10-15 Lx) during the active phase. Animal tracks were recorded by a digital video camera mounted above the maze and connected to a computer running a tracking-software (ANY-maze Video Tracking System v.4.99 – Stoelting Co., Wood Dale, IL, USA).

Open Field Test (OFT)

Rats were placed in a round arena (140 cm of diameter, 30 cm of depth) and their motor and exploratory activities were monitored for 60 min.

Elevated Plus Maze (EPM)

The elevated plus maze consisted of two opposite open arms (50cm L x 10cm W x 42.5cm H) and two opposite closed arms (50cm L x 10cm W) arranged in a cross and elevated 50 cm above the floor. In the center, a small platform (10cm x 10cm) gave access to all arms. Rats were gently placed in the center of the maze face to a close arm and their behavior was monitored for 5 min. The time spent on open arms was used as an index of anxiety.

Animal's training for alcohol self-administration

Laboratory rodents do not voluntarily consume alcohol to intoxication, in part because of taste aversion. Higher levels of consumption could be achieved by masking the taste of alcohol with saccharine (Roberts, Heyser, & Koob, 1999), which was faded out as alcohol concentrations increased (Dayas, Liu, Simms, & Weiss, 2007). Rats were trained under a Fixed Ratio 1 - Time Out 4sec schedule of reinforcement for a total of 105, 30-min daily sessions (25 sessions of saccharine fading + 80 sessions of ethanol self-administration). During these baseline conditions, pressing the right (active) lever delivered 0.1 mL of ethanol (10%w/v in tap water, prepared from a 94% (vol/vol) ethanol solution) in the delivery section and illuminated the diode above the active lever. The left lever was inactive, presses were recorded but had no consequence.

Screening for addiction-like behavior

Between test sessions aiming at scoring addiction-like behaviors (3 daily consecutive sessions each time), rats underwent 2 consecutive sessions of basic training during which they were trained again under the same baseline conditions (Jadhav et al., 2017).

A) Inability to abstain during a signaled period of reward unavailability

Rats underwent 3 daily consecutive sessions; each one consisted of an 8 minute-period of reward availability, followed by a 4 minute-period of signaled unavailability. Repeated three times, this sequence resulted in a total of 36 min. The period of unavailability was signaled by lighting up the self-administration chamber house light and interrupting the cubicle fan. The light diode above the active lever remained off after lever presses, and alcohol was not delivered. The average number of active lever presses during the signaled unavailability periods indicated the persistence in drug seeking during the no drug period.

B) High motivation for alcohol seeking

Rats were required to progressively increase the number of active lever presses between two successive rewards based on the progression sequence given by the following formula: response ratio = (5e(reward× 0.2)) − 5. Hence, the progressive-ratio schedule followed the progression: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95,

118, etc. Each session lasted 90 min, or automatically shut down following 20 consecutive minutes of inactivity on the active lever. The maximal number of active lever presses performed to reach the final ratio was defined as the breakpoint, a value reflecting animals' motivation to get the reward. The breaking point across 3 consecutive daily sessions was averaged and considered as marker of motivation for alcohol seeking.

C) Resistance to punishment

In this paradigm, each lever press delivered 0.1 mL of 10% w/v ethanol by means of a dipper, followed by mild electric foot shocks (0.22 mA for 0.5 second) through the grid of the SA chamber when the dipper retracted. This was conducted for 3 consecutive daily sessions, and the average number of active lever presses across these 3 consecutive trials was considered as a marker of resistance to punishment, reflecting a compulsive reward seeking and taking behavior.

Selection of resilient rats for microbiome analysis

We intended to select the rats falling at the lower end of the resilient group, meaning not only they would have 0 criterion, but most importantly their lever pressing behavior was constantly low (as compared to rats near the inclusion condition for each criterion). Therefore, we took the data of 40 Resilient rats and identified which rats fall in the lower 50 percentile for each of the 3 behaviours (yellow boxes in the table). Then we selected those rats which fell in the lowest 50 percentile for 2 or 3 behaviours (red boxes in

table). Most of the rats selected by this method belonged to the original 0 Criterion group, except 2 rats which belonged to the 1 Crit group (rat#51 and #54). However, one of this 1 Crit rat had to be isolated in a single cage towards the end of the experiment since it displayed aggressive behaviour. Hence, this rat was not selected and thus we ended up with 18 0 Crit rats and one, 1Crit rat of the resilient group. We considered rat#51 (1Crit) had to be included in the selection of resilient rats for microbiome analysis given its limited lever pressing behavior in general, even though it reached the inclusion criterion for the progressive ratio experiment.

Rats	A	B	C	Nb of Criterion
1	5,67	27,33	10,00	0
3	9,33	40,33	7,67	0
4	11,67	37,67	3,67	1
5	4,33	28,67	8,00	0
6	5,67	21,00	5,33	0
7	3,33	28,33	6,00	0
13	5,33	35,00	3,00	0
15	8,33	28,33	11,00	0
16	4,67	39,00	1,67	0
17	9,33	28,00	9,00	0
18	2,33	23,67	3,33	0
19	8,00	16,00	5,67	0
20	9,00	17,67	12,33	0
21	11,00	31,33	20,33	1
22	8,67	26,33	5,00	0
23	6,00	34,33	5,33	0
24	6,00	15,33	3,67	0
26	5,00	30,00	24,33	1
28	15,00	37,67	20,67	1
30	6,67	40,67	24,33	1
35	9,00	35,00	21,67	1
36	7,67	46,33	8,33	1
38	6,00	32,33	36,67	1
39	8,33	40,33	50,67	1
42	7,67	25,67	10,67	0
44	7,33	19,67	10,00	0
45	8,00	28,00	4,67	0
46	2,33	14,33	4,00	0
47	2,00	21,67	4,00	0
48	6,00	34,00	6,33	0
49	5,00	24,00	9,33	0
50	6,67	31,00	5,00	0
51	3,67	46,33	4,67	1
53	10,00	25,67	12,67	1
54	5,67	22,33	38,67	1
55	6,67	25,67	5,33	0
56	3,33	32,00	13,00	0
57	6,00	19,67	8,67	0
59	9,67	37,67	11,00	1
60	8,67	57,67	19,67	1

Rat falling in the lowest 50 percentile for this criterion
All rats selected for microbiota analysis, but #54

Partial grid paradigm

In this paradigm, the rats must press the active lever once to gain access to 0.1mL of 10% ethanol. The grid was divided into thirds, 2/3rd of the grid closest to the lever and delivery section delivered shock continuously, 1/3rd of the grid farthest from the lever was not electrified. Test rats had to bear shock before and during lever presses to administer ethanol, a condition defined as a conflict model (Barnea-Ygael et al., 2012; Cooper et al., 2007). The rats were exposed to this paradigm for 30 mins for 3 sessions of increasing shock strength of 0.1mA, 0.15mA and 0.2mA. The number of active lever presses was recorded.

Reinstatement paradigm

At the end of all behavioral paradigm tests, rats were subjected to approximately 45 days of forced abstinence after which they were re-exposed to the self-administration chambers. The rats were given access to the same conditions as the training sessions, where one active lever presses resulted in lighting up of the cue light above the lever, however ethanol was not delivered. The number of active lever presses during this period of 30 minutes measured propensity to relapse.

Results

Persistence in lever pressing during the no-drug period

The mean number of lever presses for each group were 6.03 ± 0.43 (0Crit), 8.43 ± 0.79 (1Crit), 12.28 ± 1.16 (2Crit) and 12.33 ± 0.93 (3Crit), respectively. A one-way ANOVA

showed a significant difference between groups ($F_{3,55} = 17.436$, $p < 0.0001$). Post hoc Bonferroni's test revealed that, during the no-drug period, the 3Crit rats exhibited higher lever presses compared to 0Crit ($p < 0.0001$) and 1Crit ($p<0.01$) groups, but not 2Crit ($p>0.05$). The 2 criteria rats differed from 0 criteria rats ($p < 0.001$) and 1 criteria rats ($p <0.01$). The 0 and 1 criterion rats had similar performances ($p>0.05$).

Increased motivation for alcohol seeking and drinking in an effortful condition

The average breaking points for the four groups were 26.34 ± 1.37 (0Crit), 37.21 ± 2.43 (1Crit), 49.33 ± 4.06 (2Crit) and 51.57 ± 3.27 (3Crit). One-way ANOVA showed a statistically significant difference among the groups ($F_{3,55} = 23.23$, $p < 0.0001$). Post hoc Bonferroni's tests revealed that the 3Crit rats displayed an increased motivation for ethanol seeking compared to 0Crit ($p < 0.0001$) and 1crit ($p<0.01$) groups, but not compared to 2Crit ($p>0.05$). The 2Crit rats displayed a higher breaking point compared to 0Crit ($p < 0.001$) and 1Crit ($p<0.01$) groups. Finally, even the 1Crit rats exhibited a higher breaking point compared to the 0Crit rats ($p < 0.001$).

Resistance to punishment

The average lever presses for each group when each lever press was associated with a mild shock were 6.84 ± 0.6 (0Crit), 21.23 ± 3.6 (1Crit), 20.36 ± 3.58 (2Crit) and 43.62 ± 7.28 (3Crit) (Fig. 2D). One-way ANOVA showed a statistically significant difference among the groups ($F_{3,55} = 22.55$, $p < 0.0001$). Post hoc Bonferroni's tests that 3Crit rats accepted more shocks than 2Crit ($p<0.0001$), 1Crit ($p < 0.0001$), and 0Crit rats ($p<0.01$). The 2Crit rats had higher lever presses as compared to 0Crit rats

($p<0.001$), but not 1Crit rats ($p>0.05$). Finally, even the 1Crit rats had higher lever presses than 0Crit rats ($p<0.001$).

Addiction score and vulnerability to relapse after protracted abstinence

The scores for the four groups were -2.06 ± 0.16 (0Crit), 0.23 ± 0.28 (1Crit), 2.04 ± 0.4 (2Crit) and 3.68 ± 0.6 (3Crit). A one-way ANOVA revealed a significant difference between groups ($F_{3,55} = 67.20$, $p<0.0001$). A post hoc Bonferroni's test showed that each group was significantly different every other group. Pearson's correlation analysis showed that the three criteria scores were highly correlated with the addiction score (persistence in drug seeking in absence of alcohol [$r=0.77$, $p<0.0001$], excessive motivation for alcohol seeking and drinking [$r= 0.836$, $p<0.0001$] and resistance to punishment [$r=0.536$, $p<0.0001$]).

Rats were subjected to a 45-day period of forced abstinence, followed by which the rats were exposed to the reinstatement paradigm. A one-way ANOVA revealed a significant difference between criterion groups ($F_{3,55}=6.13$, $p<0.01$). A post hoc Bonferonni's test showed that the 2Crit and 3Crit rats had higher active lever presses compared to 0Crit ($p<0.01$) and 1Crit ($p<0.001$).

Once the four groups were identified, we analyzed their lever pressing behavior during the training sessions.

	Session 6-10	Session 21-25	Session 41-45	Session76-80
0Crit	39.20 ± 3.59	30.26 ± 2.10	30.56 ± 1.88	43.67 ± 2.74
1Crit	41.40 ± 4.48	37.77 ± 2.58	44.82 ± 4.64	46.31 ± 3.08
2Crit	35.25 ± 2.95	37.86 ± 3.59	37.41 ± 3.62	49.32 ± 4.78
3Crit	41.05 ± 3.44	41.17 ± 3.16	50.40 ± 3.69	64.46 ± 2.94

Table1: Evolution of lever pressing behavior over the course of the operant conditioning

A two-way repeated measures ANOVA with repetition in one factor identified a significant group effect ($F_{3,55} = 4.93$, $p=0.0042$), a significant effect of number of training sessions ($F_{3,177} = 14.03$, $p<0.001$) and a significant effect of group X number of training session interaction ($F_{9,165} = 2.05$, $p=0.0361$). A post hoc Bonferroni's test to compare the difference between subjects at a particular training session revealed that all the groups were comparable to each other at training session 6-10 and training sessions 21-25. The number of lever presses between groups were significantly different at session 41-45 (0crit vs 3crit, $p=0.0004$). Also, the number of lever presses between groups were significantly different at session 76-80 (0crit vs 3 Crit, $p=0.0007$ and 1crit vs 3crit, $p= 0.0053$). A post hoc Bonferroni's test to compare the differences within a group of rats, showed that for all the groups the active lever presses at session 76-80 were significantly higher than the lever presses at session 6-10 ($p<0.01$) and at session 21-25 ($p<0.01$). This shows at all the groups showed a steady increase in their

lever pressing behavior as compared to their baseline lever pressing behavior. However, since the interaction effect is significant, it shows that magnitude of change was higher in the 2crit and 3crit rats.

	Average active lever presses for saccharine only	**Average active lever presses for ethanol only (Session 6-10)**	**Average active lever presses for ethanol only (Session 41-45)**	**Average active lever presses for ethanol only (Session 76-80)**
Persistence in drug seeking during the no-drug period	-0.04	-0.085	0.23	0.206
Excessive motivation for alcohol seeking and drinking	0.126	-0.013	0.497*	0.446*
Resistance to punishment	0.192	0.062	0.536*	0.27*

Table 2: *$p < 0.05$ Significant using Pearson's correlational analysis (2 tailed).

One argument against the model could be that some rats are inherently good at pressing the lever and the final output observed with the 3 behaviours reflects their motor abilities and not their conditioning for lever pressing for alcohol. Hence, we ran a correlational analysis between the three criteria scores used to define the addiction vulnerability and the lever presses for saccharine and for ethanol (at 3 time points: session 6-10, session 41-45 and session 76-80). As can be seen from the table 2, the lever presses for saccharine were not correlated with the three criteria scores. Similarly, the lever pressing for ethanol at the beginning of the training did not correlate with the

three criteria scores. The persistence in lever pressing during no-drug periods never correlated with the lever pressing at any time points. The motivation and resistance to punishment started showing significant correlations with the lever pressing for ethanol only after prolonged training.

Effect of Baclofen

Baclofen, a GABA-B receptor agonist, was tested on the progressive ratio paradigm to determine whether it could decrease the motivation for ethanol (Figure 1I). Two doses of baclofen were tested (1mg/kg and 2 mg/kg). A two-way repeated measures ANOVA with repetition in one factor identified a significant group effect ($F_{1,56} = 11.09$, $p<0.01$), a significant effect of dosage ($F_{2,116} = 25.71$, $p<0.0001$) and a significant interaction effect ($F_{112,173} = 10.46$, $p<0.0001$). A group-wise post hoc Bonferroni's test showed that both the doses of baclofen reduced ethanol intake in the Vulnerable group, while only the 2 mg/kg dose had an effect in the Resilient group. This observation is line with previous reports indicating that the reinforcing and motivational properties of alcohol in different lines of alcohol-preferring rats are differentially sensitive to treatment with baclofen (Maccioni et al., 2012).

Additionally, the effect of baclofen in the reinstatement paradigm was tested using the more effective 2mg/kg dosage (Figure 1J). A two-way repeated measures ANOVA with repetition in one factor identified a significant group effect ($F_{1,55} = 9.25$, $p<0.01$), a significant effect of intensity of dose of baclofen ($F_{1,55} = 67.81$, $p<0.0001$) and a significant interaction effect ($F_{55,113} = 7.55$, $p<0.01$).

Factor analysis-figure

A factor analysis was conducted for the three addiction-like criteria, reinstatement and impulsivity to determine whether they loaded on the same underlying construct (Figure 1L). The eigenvalue was kept as 1. The sampling adequacy score were also all around 0.75 as measured by the KMO test (Persistence in drug seeking during the no-drug period: 0.768, Excessive motivation for alcohol seeking: 0.732, Resistance to punishment: 0.793, reinstatement: = 0.786 and impulsivity: 0.802) which indicates that five variables included are suited for testing factor analysis. The five included variables loaded on one construct accounting for 50% of the variance, further supporting that the three addiction-like criteria, reinstatement and the preexisting trait of impulsivity are measures of a single underlying factor:

- Persistence in drug seeking during the no-drug period: r = 0.719
- Excessive motivation for alcohol seeking: r = 0.832
- Resistance to punishment: r = 0.715
- Reinstatement: r = 0.658
- Impulsivity: r=0.65

References

Barnea-Ygael N, Yadid G, Yaka R, Ben-Shahar O, Zangen A. (2012) Cue-induced reinstatement of cocaine seeking in the rat "conflict model": effect of prolonged home-cage confinement. Psychopharmacology, 219(3):875-883

Cooper A, Barnea-Ygael N, Levy D, Shaham Y, Zangen A. (2007) A conflict rat model of cue-induced relapse to cocaine seeking. Psychopharmacology, 194(1):117-125.

Dayas CV, Liu X, Simms JA, Weiss F. (2007) Distinct patterns of neural activation associated with ethanol seeking: effects of naltrexone. Biological Psychiatry, 61(8):979-989.

Maccioni P, Zaru A, Loi B, Lobina C, Carai MA, Gessa GL, Capra A, Mugnaini C, Pasquini S, Corelli F, Hyytiä P, Lumeng L, Colombo G. (2012) Comparison of the effect of the GABAB receptor agonist, baclofen, and the positive allosteric modulator of the GABAB receptor, GS39783, on alcohol self-administration in 3 different lines of alcohol-preferring rats. Alcohol Clin Exp Res, 36(10):1748-1766.

Roberts AJ, Heyser CJ, Koob GF. (1999) Operant self-administration of sweetened versus unsweetened ethanol: effects on blood alcohol levels. Alcohol Clin Exp Res, 23(7):1151-1157.

Microbiome and Behavior Significant Correlations				RAW	Bayesian
Taxa	**Behavior**	**Category**	**rho**	**P value**	**Q value**
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.uncultured	PR	AUD Behaviour	-0.5781	0.000144	0.012371
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Intestinimonas	LP.wShock	AUD Behaviour	0.574573	0.000162	0.012371
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Shuttleworthia	TotEtOH	Behaviour	-0.54853	0.000363	0.018531
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.006	D2R	Dorsal Striatum mRNA	-0.78571	0.000516	0.019771
Firmicutes.Bacilli.Bacillales.Family.XI.Gemella	LP.wShock	AUD Behaviour	-0.52572	0.0007	0.021434
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.uncultured	Addiction.score	AUD Severity	-0.47959	0.002313	0.044387
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Anaerofilum	Reinstatement	Relapse Behaviour	-0.47949	0.002319	0.044387
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.005	LP.woEtOH	AUD Behaviour	0.482393	0.002161	0.044387
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.rumen.bacterium	PR	AUD Behaviour	0.470505	0.002873	0.048879
Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrio	LP.woEtOH	AUD Behaviour	-0.45808	0.003828	0.054802
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Intestinimonas	Addiction.score	AUD Severity	0.456833	0.003937	0.054802
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.007	OFT	Behaviour	-0.44425	0.005877	0.058619
Firmicutes.Bacilli.Bacillales.Family.XI.Gemella	Criteria	AUD Severity	-0.44089	0.005596	0.058619
Firmicutes.Bacilli.Bacillales.Family.XI.Gemella	PR	AUD Behaviour	-0.43581	0.006239	0.058619
Firmicutes.Bacilli.Bacillales.Family.XI.Gemella	Addiction.score	AUD Severity	-0.4305	0.006977	0.058619
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Anaerofilum	EPM	Behaviour	-0.42855	0.00649	0.058619
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.uncultured	Criteria	AUD Severity	-0.42137	0.008422	0.058619
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Intestinimonas	Criteria	AUD Severity	0.422197	0.008281	0.058619
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.rumen.bacterium	Criteria	AUD Severity	0.422541	0.008223	0.058619
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.uncultured	LP.wShock	AUD Behaviour	0.423894	0.007999	0.058619
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.uncultured	Addiction.score	AUD Severity	0.430791	0.006934	0.058619
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.uncultured	LP.woEtOH	AUD Behaviour	0.434307	0.00644	0.058619
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.006	EPM	Behaviour	0.410446	0.00945	0.062917
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Intestinimonas	PR	AUD Behaviour	0.41265	0.010034	0.063963
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Shuttleworthia	D2R	Dorsal Striatum mRNA	0.635714	0.010861	0.063963

Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Syntrophococcus	D2R	Dorsal Striatum mRNA	0.635714	0.010861	0.063963
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.uncultured	LP.wShock	AUD Behaviour	-0.40035	0.012751	0.069734
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.uncultured	5CSRTT	Behaviour	0.395725	0.012645	0.069734
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Eubacterium.coprostanoligenes.group	OFT	Behaviour	-0.40161	0.013748	0.072591
Firmicutes.Bacilli.Bacillales.Family.XI.Gemella	D1R	Dorsal Striatum mRNA	-0.61419	0.014854	0.075819
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.rumen.bacterium	LP.woEtOH	AUD Behaviour	0.38807	0.016062	0.079338
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Hydrogenoanaerobacterium	LP.wShock	AUD Behaviour	-0.37932	0.018838	0.083461
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.rumen.bacterium	Addiction.score	AUD Severity	0.378903	0.01898	0.083461
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.006	LP.wShock	AUD Behaviour	0.38064	0.018396	0.083461
Firmicutes.Bacilli.Bacillales.Family.XI.Gemella	D2R	Dorsal Striatum mRNA	0.595854	0.019077	0.083461
Firmicutes.Bacilli.Bacillales.Family.XI.Gemella	TotEtOH	Behaviour	-0.37384	0.020773	0.083706
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Eubacterium.oxidoreducens.group	LP.woEtOH	AUD Behaviour	0.375267	0.020255	0.083706
Firmicutes.Erysipelotrichia.Erysipelotrichales.Erysipelotrichaceae.Allobaculum	D2R	Dorsal Striatum mRNA	0.591271	0.020263	0.083706
Firmicutes.Negativicutes.Selenomonadales.Veillonellaceae.Veillonella	D1R	Dorsal Striatum mRNA	-0.58557	0.021817	0.084913
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Hydrogenoanaerobacterium	D1R	Dorsal Striatum mRNA	-0.58352	0.022399	0.084913
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.rumen.bacterium	D2R	Dorsal Striatum mRNA	-0.57857	0.023847	0.084913
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Papillibacter	D1R	Dorsal Striatum mRNA	-0.57143	0.026063	0.084913
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.007	Reinstatement	Relapse Behaviour	-0.36639	0.023671	0.084913
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Hydrogenoanaerobacterium	Addiction.score	AUD Severity	-0.36518	0.02417	0.084913
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.006	Criteria	AUD Severity	0.362255	0.025416	0.084913
Firmicutes.Erysipelotrichia.Erysipelotrichales.Erysipelotrichaceae.Erysipelotrichaceae.UCG.003	D2R	Dorsal Striatum mRNA	0.571726	0.025968	0.084913

Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Hydrogenoanaerobacterium	D2R	Dorsal Striatum mRNA	0.575986	0.024632	0.084913
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.NK4B4.group	LP.woEtOH	AUD Behaviour	0.359166	0.02679	0.085463
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Eubacterium.coprostanoligenes.group	5CSRTT	Behaviour	-0.35044	0.02873	0.089202
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.005	5CSRTT	Behaviour	0.349628	0.029127	0.089202
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Papillibacter	EPM	Behaviour	0.346981	0.030454	0.091437
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Anaerofilum	D2R	Dorsal Striatum mRNA	0.553571	0.032287	0.095076

Table 3: Significant correlations between microbiome and behavior for Figure 6 of manuscript.

Significant correlated genera presence and abundance	Relative Abundance (%)		All Samples		Resilient	Vulnerable
Taxa	Median	Mean	Presence	Presence%	Presence	Presence
Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrio	1.076%	1.569%	38	100.0%	19	19
Firmicutes.Negativicutes.Selenomonadales.Veillonellaceae.Veillonella	0.000%	0.012%	10	26.3%	7	3
Firmicutes.Erysipelotrichia.Erysipelotrichales.Erysipelotrichaceae.Erysipelotrichaceae.UCG.003	0.002%	0.005%	26	68.4%	13	13
Firmicutes.Erysipelotrichia.Erysipelotrichales.Erysipelotrichaceae.Allobaculum	0.000%	0.010%	19	50.0%	11	8
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Papillibacter	0.123%	0.130%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Intestinimonas	0.103%	0.108%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Hydrogenoanaerobacterium	0.000%	0.001%	18	47.4%	9	9
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Eubacterium.coprostanoligenes.group	2.351%	2.893%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Anaerofilum	0.006%	0.007%	35	92.1%	19	16
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.uncultured	4.017%	4.539%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Syntrophococcus	0.006%	0.010%	35	92.1%	18	17
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Shuttleworthia	0.014%	0.028%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.007	0.001%	0.002%	24	63.2%	12	12
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.006	0.077%	0.083%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.005	0.142%	0.178%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.NK4B4.group	0.016%	0.025%	37	97.4%	18	19
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Eubacterium.oxidoreducens.group	0.125%	0.155%	38	100.0%	19	19
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.rumen.bacterium	0.009%	0.018%	34	89.5%	15	19
Firmicutes.Bacilli.Bacillales.Family.XI.Gemella	0.000%	0.001%	9	23.7%	8	1
Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.uncultured	0.109%	0.121%	38	100.0%	19	19

Table 4: Relative abundance and sample presence of genus level bacteria shown in correlation results - Figure 6 of manuscript.

Taxa	Behavior	rho	P value	Q value
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	LP.wShock	-0.52572	0.0007	0.031114
D_0__Bacteria.D_1__Proteobacteria.D_2__Deltaproteobacteria.D_3__Desulfovibrionales. D_4__Desulfovibrionaceae	LP.woEtOH	-0.46947	0.002944	0.079496
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__4.15.D_4__uncultured.bacterium	TotEtOH	0.443216	0.005322	0.121537
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	Criteria	-0.44089	0.005596	0.124331
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	PR	-0.43581	0.006239	0.132315
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	Addiction score	-0.4305	0.006976	0.140972
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	D1.mRNA	-0.61419	0.014854	0.222395
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Peptococcaceae	OFT	-0.39562	0.016925	0.240916
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Defluviitaleaceae	dPR. baclofen	0.383965	0.017318	0.244105
D_0__Bacteria.D_1__Firmicutes.D_2__Negativicutes.D_3__Selenomonadales.D_4__Acidaminococcaceae	Addiction score	-0.38047	0.018452	0.254555
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	D2.mRNA	0.595854	0.019077	0.255258

Table 5: Family level correlations to behavioral measures. Green highlights indicate correlations that pass FDR with a q value set to 0.25.

	Relative Abundance (%)		All Samples		Resilient	Vulnerable
Taxa	Median	Mean	Presence	Presence%	Presence	Presence
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__Bacillales.D_4__Family.XI	0.000%	0.001%	9	23.684%	8	1
D_0__Bacteria.D_1__Proteobacteria.D_2__Deltaproteobacteria.D_3__Desulfovibrionales.D_4__Desulfovibrionaceae	1.189%	1.714%	38	100.000%	19	19
D_0__Bacteria.D_1__Firmicutes.D_2__Bacilli.D_3__4.15.D_4__uncultured.bacterium	0.000%	0.001%	10	26.316%	3	7
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Peptococcaceae	0.737%	0.752%	38	100.000%	19	19
D_0__Bacteria.D_1__Firmicutes.D_2__Clostridia.D_3__Clostridiales.D_4__Defluviitaleaceae	0.004%	0.004%	32	84.211%	15	17

Table 6: Relative abundance and sample presence of family level bacteria shown in correlation results - Table 5 above.