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ORIGINAL ARTICLE

Alterations in the Gut Microbiota of Rats Chronically Exposed to Volatilized Cocaine and Its Active Adulterants Caffeine and Phenacetin

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Abstract
A role of the gut microbiota in influencing brain function and emotional disorders has been suggested. However, only a few studies have investigated the gut microbiota in the context of drug addiction.

Cocaine can be smoked (i.e., crack or coca paste) and its consumption is associated with a very high abuse liability and toxicity. We have recently reported that cocaine base seized samples contained caffeine and phenacetin as main active adulterants, which may potentiate its motivational, reinforcing, and toxic effects. However, the effect of volatilized cocaine and adulterants on the gut microbiota remained unknown. In the present study, we evaluated the effect of volatilized cocaine and two adulterants on the structure, diversity, and functionality of the gut microbiota in rats. Animals were chronically exposed to the fume of cocaine, caffeine, and phenacetin during 14 days. At the end of the treatment, feces were collected and the structure, composition, and functional predictions of the gut microbiota were analyzed. Cocaine significantly decreased the community richness and diversity of the gut microbiota while both cocaine and phenacetin drastically changed its composition. Phenacetin significantly increased the Firmicutes-Bacteroidetes ratio compared to the control group. When the predicted metagenome functional content of the bacterial communities was analyzed, all the treatments induced a dramatic decrease of the aromatic amino acid decarboxylase gene. Our findings suggest that repeated exposure to volatilized cocaine, as well as to the adulterants caffeine and phenacetin, leads to changes in the gut microbiota. Future studies are needed to understand the mechanisms underlying these changes and how this information may support the development of novel treatments in drug addiction.

Keywords Gut-brain axis · Cocaine · Adulterants · Addiction · Microbiota

Introduction

Compelling evidence indicates that the gut microbiota is intimately involved in several physiological aspects, from the nutritional status to behavior and response to stress (Kim et al. 2013; Rieder et al. 2017; Sekirov and Finlay 2009). The human gut microbiota is composed of Bacteroidetes and Firmicutes and to a lesser extent of the phyla Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia, among others (Grenham et al. 2011). Particularly, the relative abundance of Firmicutes and Bacteroidetes and the Firmicutes/Bacteroidetes ratio have been frequently regarded as relevant in the human gut microbiota composition. The Firmicutes/Bacteroidetes ratio has been related with different health conditions like obesity, inflammatory bowel diseases, autism spectrum disorders, and even age, among others (Mathur and Barlow 2015; Chung et al. 2016; Strati et al. 2017; Pérez-Martínez et al. 2014). Similarly, the rat microbiota contains the same dominant bacteria phyla in the gastrointestinal tract, with Firmicutes and Bacteroidetes representing also the largest proportions (Zhu et al. 2016).

It has also been proved that the disruption of the normal structure of the gut microbiota (dysbiosis) induced by

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different factors may be associated with several neuropsychiatric disorders (Dinan and Cryan 2017; Rieder et al. 2017). This association endorses the influence of a bidirectional pathway of communication along the microbiota-gut-brain axis (Cryan and Dinan 2012; Wiley et al. 2017). Within this communication, a pathway for the reductive metabolism of aromatic amino acids by *Clostridium sporogenes* and *Ruminococcus gnavus* (two members of the Firmicutes phylum) has been reported by Williams and colleagues (Williams et al. 2014). These authors found that these species were capable of decarboxylating tryptophan to tryptamine, an extremely rare activity among bacteria. They also showed that at least 10% of the human metagenomes harbor one of these enzymes, a fact that could lead to a new road to explore the role of this function in the microbe-host interaction (Williams et al. 2014). Interestingly, if the tryptamine production represents a microbiota-mediated alteration in tryptophan metabolism (Williams et al. 2014), this indirectly could imply an influence in the production of serotonin in the brain, one of the main neurotransmitter related to emotion and mood disorders. Several findings on animals and humans support the role of microbiota-gut-brain connection on mood-, anxiety-, and autism-related disorders (Buffington et al. 2016; Crumeyrolle-Arias et al. 2014). However, there has been little research carried out to elucidate a possible link between alcohol and drug addiction and the gut microbiota (for a recent review, see Temko et al. 2017). Only two studies have reported so far different aspects of cocaine consumption and effects on the gut microbiota. A human study identified differences in the relative abundance of the major phyla of the intestinal microbiota of cocaine users and HIV-infected patients (Volpe et al. 2014). Cocaine users and HIV-positive cocaine users exhibited a higher relative abundance of Bacteroidetes than non-users. In this study, it is suggested that several other factors such as nutrition, alcohol use, and lean body mass may contribute to the observed differences, therefore raising the question whether cocaine itself may have an impact on the gut microbiota (Volpe et al. 2014). Using a more controlled experimental approach, Kiraly and colleagues (Kiraly et al. 2016) reported that the treatment of mice with oral antibiotics resulted in a reduction of gut bacteria and an increased sensitivity to cocaine reward as well as an enhanced sensitivity to the locomotor sensitizing effects of repeated cocaine administration. This study represents the first piece of evidence that manipulations of the gut microbiota affect behavioral response to cocaine (Kiraly et al. 2016).

Cocaine can be consumed by snorting as cocaine hydrochloride or by pulmonary inhalation as cocaine freebase. Crack and coca paste (CP) are the best known smokable forms of cocaine (Hatsukami and Fischman 1996; López Hill et al. 2011). In comparison to cocaine hydrochloride, the chronic pulmonary inhalation of cocaine induces a faster and higher degree of dependence in users among other physiological and behavioral alterations (Gossop et al. 1992; Hatsukami and Fischman 1996). The factors responsible for this high abuse liability are not fully understood. However, some clinical and preclinical evidence supports that the route of administration (i.e., pulmonary inhalation is considered a very fast route of administration like the intravenous route), cocaine purity, and drug adulteration with other active substances could explain it (Abin-Carriquiry et al. 2018; Broseus et al. 2016; Gossop et al. 1992; Prieto et al. 2015, 2016; Samaha and Robinson 2005). Forensic data of cocaine base seized samples (i.e., CP) have shown that caffeine and phenacetin are the most frequently used substances as adulterants (Abin-Carriquiry et al. 2018). Using different animal models, we have demonstrated that caffeine potentiates the stimulant (López Hill et al. 2011), the rewarding and the motivational effects of cocaine (Prieto et al. 2015, 2016). On the other hand, less preclinical evidence has been reported about phenacetin (an analgesic and antipyretic drug) since its role as an adulterant is controversial due to its high toxicity (Villar Núñez et al. 2018).

Considering a possible link between drug addiction and the gut microbiota (Kiraly et al. 2016; Ning et al. 2017; Volpe et al. 2014; Xiao et al. 2018), we hypothesized that the diversity and structure of the intestinal microbiota of rats chronically treated with volatilized cocaine base and main adulterants (caffeine and phenacetin) would be affected. The aim of the present study was to characterize the effect induced by the chronic exposition to volatilized cocaine, caffeine, and phenacetin, on the diversity and abundance of the rat gut microbiota. A predictive analysis of the metagenome functional content of the fecal microbiota was also performed.

Material and Methods

Animals and Housing Conditions

Twelve adult male Wistar rats weighing 250–310 g were used in this study. The groups of rats were generated using animals that came from different cages before the start of the experiment. These two factors (the age of the rats and the composition of the different groups) were taken into account to minimize a possible cage effect on the microbiota (Laukens et al. 2016). Animals were exposed to identical environmental conditions such as food and water available ad libitum and kept under controlled conditions (temperature 22 ± 2 °C, 12-h light-dark cycle, lights on at 7:00 A.M.). Along the experiment, these controlled conditions were kept and stressors such as noise and handling by different persons were avoided. The experimental groups (3 animals each) were housed in the same type of cage (50 × 37.5 × 21 cm), in the same rack and in the same room of the facility. All procedures were

carried out in accordance with the Instituto de Investigaciones Biológicas Clemente Estable (IIBCE) Bioethics Committee's requirements, consistent with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978), and under the current ethical regulations of the national law on animal experimentation no. 18.611. Adequate measures were taken to minimize discomfort or stress of the animals, and all efforts were made to use the minimal number of animals necessary to produce reliable scientific data.

Drugs and Doses

Cocaine (freebase) highly purified was generously donated by Dr. Ignacio Carrera from the School of Chemistry, Universidad de la República (Uruguay) while caffeine and phenacetin were obtained from Sigma-Aldrich (Germany). Doses of 25 mg of each drug were tested. This dose was chosen according to previous studies in which volatilized cocaine base (taken as a psychostimulant drug standard), caffeine, and phenacetin were tested in rats (Galvalisi et al. 2015; Galvalisi et al. 2017; Abin-Carriquiry et al. 2018).

Drug Volatilization and Experimental Protocol

The method applied to volatilize drugs reproduces in the lab the procedure used by humans to smoke crack or CP. The volatilization device and administration methods have already been reported in previous studies (Abin-Carriquiry et al. 2018; Galvalisi et al. 2015; Galvalisi et al. 2017). A total of 12 animals were randomly assigned to four experimental groups exposed for 14 days (Zhang et al. 2016) to either cocaine (25 mg/daily), caffeine (25 mg/daily), or phenacetin (25 mg/daily) group. The control group was subjected to the volatilization procedure and only the steel wool was heated. The daily exposure to either condition lasted 10 min. After each daily exposure procedure, the rats were removed from the inhalation chamber and returned to the original cages. On the last day of the treatment, animals were placed in a clean cage immediately after the last drug volatilization session; the feces were collected and stored at − 80 °C until the intestinal microbiota analysis was performed. Neither body weight decrement, stress, nor aggression reactions were observed along the experiment.

DNA Extraction and 16S rRNA Gene Sequencing

Aliquots of 250–300 mg of fecal pellets were transferred to clean and sterile microtubes and DNA was extracted using the FastDNA™ spin kit (MP Biomedicals) according to the manufacturer instructions. After extraction, the concentration and purity of DNA were spectrophotometrically determined at 260 and 280 nm (NanoDrop). Bacteria communities were barcoded and identified based on the ribosomal DNA (16S rRNA) sequencing (Macrogen). The sequencing libraries were prepared according to the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V3 and V4 regions. The DNA quantity was measured by PicoGreen and input gDNA (10 ng) amplified by PCR.

The primer sequences used for the first amplifications were as follows: V3-F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and V4-R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′.

The final purified product was then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the TapeStation DNA screentape (Agilent Technologies, Waldbronn, Germany). Then, the paired-end (2 × 300 bp) sequencing was performed by the Macrogen using the MiSeq™ platform (Illumina, San Diego, USA). Paired-end reads were joined using join_paired_ends.py of QIIME using the fastq_join method of QIIME (Kuczynski et al. 2011). Chimera reads were detected using vsearch (Rognes et al. 2016) and quality filtered using the split_library_fastq.py script of QIIME. Sequences were then concatenated in a single file and further analyzed using QIIME version 1.9.0.

Analyses of the Amplicon Data

To assign operational taxonomic units (OTUs) at 97% identity and to perform taxonomic classification, we used UCLUST and the SILVA_128_QIIME_release via the QIIME pick_otus.py script for alpha and beta diversity analyses; and pick_closed_reference_otus.py script and Greengenes version 13_8 reference database to create the OTU table for PICRUSt (Langille et al. 2013) for in silico metagenomic inference. Alpha diversity was analyzed through rarefaction curves using the alpha_rarefaction.py script (100,000 sequences upper limit) and alpha_diversity.py (Caporaso et al. 2010). Several indices of diversity such as Chao1, Shannon, Dominance (1-Simpson index), and phylogenetic diversity (PD) were calculated.

Bray Curtis based non-metric multidimensional scaling (nmMDS) plots were calculated to visualize the differences between the bacterial communities (beta diversity), while permutational ANOVA (PERMANOVA) was used to test for significant differences between factors of the study design (control, cocaine-, caffeine-, and phenacetin-treated rats). Weighted unifrac was used to test if the phylogenetic lineages between treatments were significantly different (Lozupone et al. 2006). Beta diversity community analyses were performed using the Past v1.77 software (Hammer et al. 2001) and beta_significance.py script of QIIME (Caporaso et al. 2010).

To identify the OTUs (bacterial species) that were most important to create the observed pattern, a SIMPER analysis was carried out (Clarke 1993). Two-way ANOVA was applied to analyze the observed differences in the abundance of the different bacterial phyla and classes between the treatments. Statistical analysis was performed using GraphPad Prism 6.0 software.

Functional Analyses

To predict the functional profile of the microbial communities from each treated rat, a PICRUSt-based approach was applied (Langille et al. 2013). This tool allows predicting the gene content of a microbial community from a 16S gene survey, giving a predicted metagenome based on the KEGG Orthology (KO) database. As it has been described, butyrate has an important role as the preferred energy source for the colonic epithelium. Thus, to evaluate the relevance of the microbial community on the gut energetics, the obtained metagenome from each sample was analyzed to look for genes involved in butyric acid synthesis (butyrate kinase, K00929, and phosphate butyryl transferase, K00634). Also, to evaluate the effect of the treatments on the community ability to convert L-tryptophan to tryptamine or catalyze the synthesis of neurotransmitters (e.g., dopamine and serotonin), the presence of genes involved in tryptophan metabolism (particularly aromatic amino acid decarboxylase, K01593) was checked in the predicted metagenome. One-way ANOVA was used to address the differences of the predicted copy number of the assessed genes between the treatments.

Results

Bacterial Richness and Diversity

According to the rarefaction curves based on the defined bacterial OTUs, the sequencing depth was satisfactory and represented the majority of bacterial species (Fig. 1a). However, the fecal bacterial community of cocaine-treated rats tended to differ from the rest, showing a diminished phylogenetic bacterial diversity (Fig. 1b; nonparametric t test and Monte Carlo simulations $p > 0.05$). Alpha diversity indexes (Chao, Shannon, Simpson Dominance, and PD indexes) were compared between the different treatments and showed that Shannon diversity was significantly lower in samples of cocaine-treated animals compared to the control group (one-way ANOVA, Bonferroni corrected $p < 0.05$, Table 1). Richness and PD values also tended to be lower in cocaine-treated rat samples than in the control group (ANOVA, $p = 0.07$). Concomitantly, the Dominance index in the microbiota of cocaine-treated rats tended to be higher than that observed in the control and the other treated groups, although the difference was not significant (one-way ANOVA, Bonferroni corrected $p > 0.05$; Table 1).

Community Composition (OTU Level) of the Fecal Microbiota in Treated Rats

To provide an overview of the gut microbiota composition of the different groups of rats, an nmMDS plot based on the OTU relative abundance was performed (Fig. 2). The distances of all points representing the relative dissimilarities of the fecal bacterial communities showed a difference between cocaine- and phenacetin-treated rats and also compared to the communities of control and caffeine-treated rats. In contrast, bacterial communities of feces of caffeine-treated and control rats were similar. PERMANOVA test showed that communities from cocaine- and phenacetin-treated rats were significantly different compared to the controls communities ($p < 0.05$; Bonferroni corrected). A weighted unifrac significance test, which is a β-diversity measure that uses phylogenetic information to compare samples, was also used. Results showed that only in cocaine- and phenacetin-treated rats, the bacterial community was significantly different in their phylogenetic composition compared to that observed in control rats ($p < 0.05$; Bonferroni corrected). Fecal bacterial communities from caffeine-treated rats were similar to those from the control group ($p > 0.05$; Bonferroni corrected).

To determine which OTUs were responsible for the observed difference between the different groups of samples, a SIMPER analysis was conducted on the OTU table. Results showed that the bacterial taxonomic composition exhibited a 60.7% average dissimilarity between treatments. We found that only 25 OTUs accounted for 20% of the observed dissimilarity between cocaine or phenacetin treatments and the control (contribution of each OTU to the observed dissimilarity ranged from 5 to 0.5%), and most of them were shared between cocaine- and phenacetin-treated rats (Table 2). Those OTUs that significantly diminished their abundance in feces of cocaine- or phenacetin-treated animals were affiliated to Desulfovibrionaceae, Spirochetaceae (*Treponema* sp.), and Ruminococcaceae families and to the Bacteroidales order. Interestingly, some OTUs belonging to this order and present in the control group in low abundance completely disappeared in the cocaine-treated rats feces together with Lactobacillaceae (*Lactobacillus* spp.), Veillonellaceae (*Quinella* spp.), and Ruminococcaceae, while presented high abundance in phenacetin-treated rats. This finding suggests that these bacterial populations would be specifically enriched in phenacetin-treated rats. Likewise, some OTUs that were present in the control group and disappeared in phenacetin-treated rat feces were present in high abundance in cocaine treatment (Table 2). These OTUs belonged to Lachnospiraceae (*Lachnoclostridium*), Prevotellaceae, and

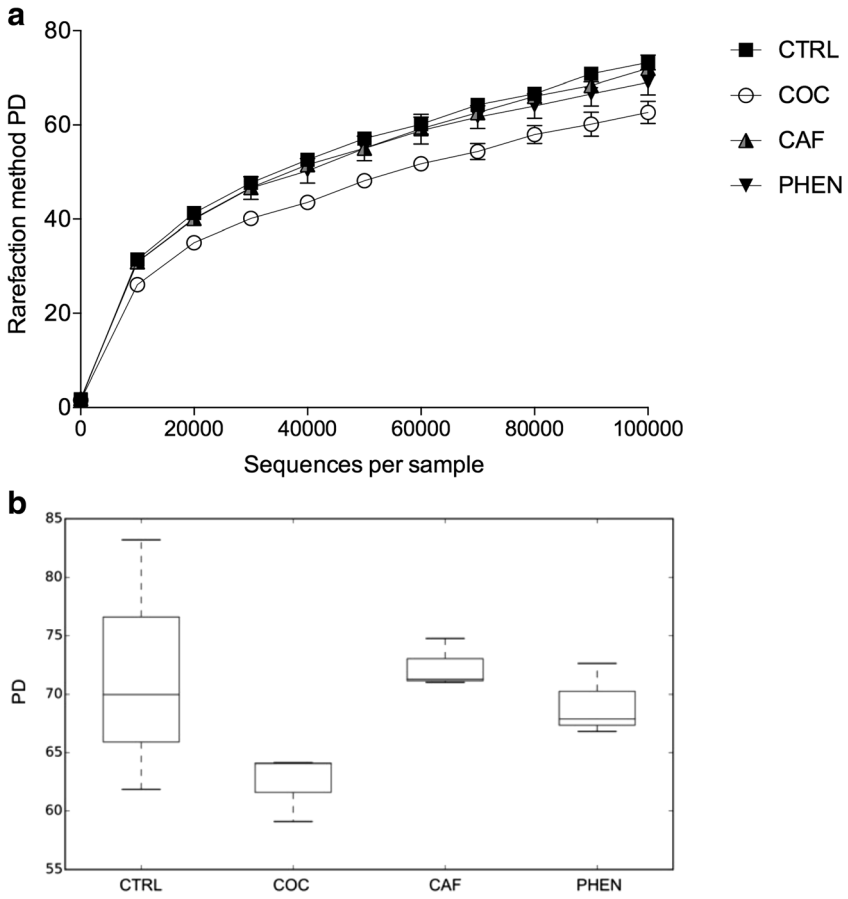

Fig. 1 Rarefaction curves of bacterial 16S rRNA gene sequences recovered from each treatment (**a**). OTUs were defined as 97% shared sequence identity. Boxplots in **b** show means, ranges, and outliers of the PD alpha diversity comparison between treatments. Sample names are as follows: sequences from control treatment, CTRL; sequences from cocaine-treated rats, COC; sequences from caffeine-treated rats, CAF; and sequences from phenacetin-treated rats, PHEN

Peptostreptococcaceae (*Peptoclostridium* spp.) and would be specifically enriched by the cocaine treatment.

Shifts on the Relative Abundance of Firmicutes and Bacteroidetes Phyla

The fecal bacterial community of the rats treated with phenacetin exhibited a significant increase in the Firmicutes/Bacteroidetes ratio related to the control group (2.32 vs. 0.93). This variation was due both to a significant increase of Firmicutes abundance and a significant decrease in the Bacteroidetes abundance ($p < 0.05$; two-way ANOVA, Bonferroni corrected; Fig. 3a). The Firmicutes/Bacteroidetes ratio in the microbiota of cocaine- and caffeine-treated rats did not show a significant variation compared to the control group. To perform a more detailed analysis on the variation of these two phyla in the phenacetin-treated rats, the bacterial classes and families accounting for those differences were analyzed. We found that in the case of the Bacteroidetes phylum, abundance of the Bacteroidia class diminished significantly after phenacetin treatment, while in the case of Firmicutes, abundance of Bacilli and Clostridia classes significantly increased ($p < 0.05$; two-way ANOVA, Bonferroni corrected). Moreover, when the variation of specific Firmicutes and Bacteroidetes families was assessed in feces of phenacetin-treated rats, it was observed that relative

Table 1 Alpha diversity indexes of the rat fecal microbiota

Alpha diversity index	Control (SD)	Cocaine (SD)	Caffeine (SD)	Phenacetin (SD)
Richness	27,624.3 (2999.5)	24,672.0 (1088.8)	27,625.0 (2442.0)	26,347.7 (2719.6)
Chao1	78,853.3 (9011.6)	70,756.8 (4022.4)	82,890.1 (12,356.7)	78,785.4 (9408.3)
PD_whole_tree	84.3 (2.9)	75.4 (5.3)	81.52 (13.2)	78.9 (3.1)
Shannon	10.3 (0.3)	9.75 (0.1)*	10.51 (0.4)	10.3 (0.2)
Dominance	0.01 (0.005)	0.02 (0.01)	0.01 (0.002)	0.01 (0.002)

Averages and standard deviation (SD) of the alpha diversity indexes calculated for bacterial communities obtained from the feces of the treated rats are shown. *The treatment that showed significant differences compared to the control ($p < 0.05$)

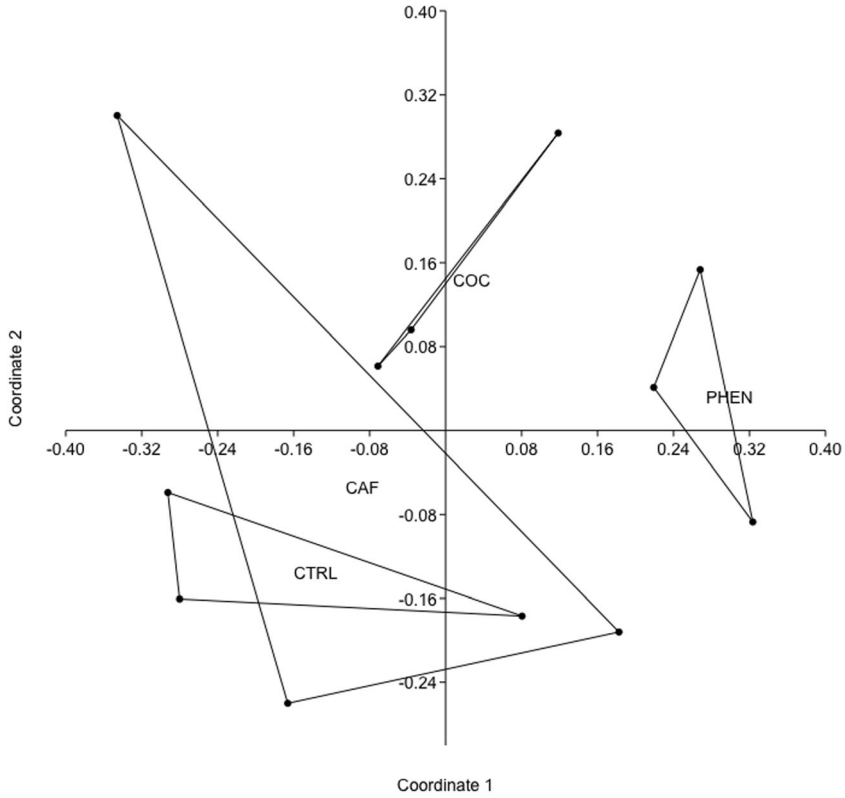

Fig. 2 Non-metric multidimensional scaling (nmMDS) based on the relative abundance of the OTUs defined as 97% shared sequence identity. Individual samples (triplicates) connected by lines (convex hull surface areas) are shown. Stress value was 0.205. Sample names were CTRL for control, COC for cocaine, CAF for caffeine, and PHEN for phenacetin treatment

abundance of Bacteroidales S24–7 and Lachnospiraceae increased while Prevotellaceae diminished ($p < 0.05$; two-way ANOVA, Bonferroni corrected). Also, abundance of members of the Ruminococcaceae family significantly decreased in feces of rats treated with cocaine ($p < 0.05$; two-way ANOVA, Bonferroni corrected).

As lactobacilli have a pivotal importance in the human gut microbiota, their relative abundance in feces was analyzed in the different groups of animals. A significant increase of lactobacilli relative abundance was observed in the feces of phenacetin-treated rats compared to the other groups ($p < 0.05$; one-way ANOVA, Bonferroni corrected; Fig. 3b).

Predicted Functional Profile of the Microbial Communities

Bioinformatics PICRUSt from the different groups of animals is shown in Fig. 4a–c. Results showed that genes involved in pathways for butyrate synthesis like butyrate kinase (K00929) significantly increased in the feces of cocaine-treated rats compared to the control group ($p < 0.05$; one-way ANOVA, Bonferroni corrected) while decreased in caffeine- and phenacetin-treated rats compared to the control group, being this decrease significant only for the caffeine treatment ($p < 0.05$; Fig. 4a). Similar results were found for the phosphate butyryl transferase (K00634), which although increased non-significantly in cocaine treatment ($p > 0.05$; one-way ANOVA, Bonferroni corrected) showed a significant decrease in caffeine- and phenacetin-treated rats compared to the controls ($p < 0.05$; one-way ANOVA, Bonferroni corrected; Fig. 4b). These findings suggest that butyrate synthesis was stimulated by the cocaine treatment. The presence and relative abundance of an aromatic amino acid decarboxylase (K01593) was also analyzed (Fig. 4c). A significant decrease induced by the three treatments was observed compared to the control animals ($p < 0.05$; one-way ANOVA, Bonferroni corrected; Fig. 4c), indicating that those bacterial populations accounting for the metabolism of tryptophan or other related neuroactive compounds (i.e., dopamine and serotonin) were altered by the three assayed treatments.

Discussion

In this study, we tested the effects induced by the chronic treatment of cocaine, caffeine, and phenacetin in rats, using the pulmonary inhalation as the route of administration, on the gut microbiota. We found that particularly cocaine and phenacetin elicited drastic changes in the gut microbiota of treated rats. Also, changes in the predicted gene content of the microbiota related to the enzymes aromatic L-amino acid decarboxylase and those involved in the butyrate synthesis pathway were detected.

Table 2 Composition of the rat fecal bacterial communities

OTU name	OTU taxonomic affiliation	Control	Cocaine	Phenacetin
denovo67138	Prevotellaceae[e]	1.2E04	1.6E04	2.3E03
denovo82695	Lachnospiraceae (*Roseburia*)[b]	301	6.2E03	6.3E03
denovo179704	Erysipelotrichaceae[b]	353	2.4E03	1.8E03
denovo74349	Desulfovibrionaceae (*Desulfovibrio*)[a]	2.1E03	104	222
denovo148929	Candidatus saccharimonas[e]	3.6E03	1.9E03	4.4E03
denovo147006	Desulfovibrionaceae (*Desulfovibrio*)[a]	1.7E03	6.3	62.3
denovo161189	Lachnospiraceae (*Lachnoclostridium*)[c]	33	1.6e3	–
denovo60133	Spirochaetaceae (*Treponema*)[a]	1.5e3	63.7	106
denovo121192	Prevotellaceae[c]	302	1.7e3	–
denovo145960	Erysipelotrichaceae (*Allobaculum*)[b]	64	1.4E03	2.0E03
denovo157972	Prevotellaceae[c]	1.2E03	2E03	–
denovo6618	Prevotellaceae (*Prevotella*)[e]	1.8E03	2.6E03	366
denovo115314	Peptostreptococcaceae (*Peptoclostridium*)[c]	511	1.6E03	–
denovo174588	Prevotellaceae (*Prevotella*)[c]	731	1.8E03	–
denovo27935	Lactobacillaceae (*Lactobacillus*)[b]	741	1.2E03	5.1E03
denovo45519	Ruminococcaceae[a,c]	1.2E03	320	–
denovo47112	Unassigned[a,c]	1.1E03	139	–
denovo47200	Lactobacillaceae (*Lactobacillus*)[b]	330	1.3E03	1.5E03
denovo115864	Lachnospiraceae[c]	755	1.5E03	–
denovo60129	Ruminococcaceae__*Eubacterium coprostanoligenes* group[c]	798	376	–
denovo39093	Bacteroidales[d]	429	–	1.9E03
denovo50498	Bacteroidales[a,d]	2.2E03	–	789
denovo47200	Lactobacillaceae (*Lactobacillus*)[D]	330	–	1.5E03
denovo30989	Veillonellaceae (*Quinella*)[d]	403	–	1.4E03
denovo182635	Ruminococcaceae[d]	651	–	1.5E03

OTUs accounting for the differences in the composition of the bacterial communities observed between the treatments (SIMPER analysis). The OTU names and their taxonomic affiliations are shown. [a] OTUs that significantly decreased in cocaine and phenacetin treatments related to the control; [b] OTUs that significantly increased in cocaine and phenacetin treatments related to the control; [c] OTUs that disappeared from phenacetin treatment; [d] OTUs that disappeared from cocaine treatment; [e] OTUs showing a slight increase either in cocaine or phenacetin related to the control

The rewarding and motivational effects induced by cocaine have been widely reported. However, the effect of cocaine on the gut microbiota is being initially studied. To date, only a single study has reported the effect of cocaine on fecal microbiota in cocaine users and non-users (Volpe et al. 2014). On the other hand, caffeine and phenacetin are the substances most frequently found in seized samples of psychostimulants, including cocaine hydrochloride and smokable forms of cocaine (Abin-Carriquiry et al. 2018; Cole et al. 2011). So far, no preclinical or clinical evidence showing the effect of these adulterants on the gut microbiota had been reported.

Changes in lifestyle, diet, and stress are directly associated with drug addiction (Ning et al. 2017; Volpe et al. 2014). However, here, we demonstrated for the first time that drug exposure per se alters the gut microbiota in rats through inhalation, a route that leads to central penetrance and facilitates a rapid development of addiction. While we cannot rule out a mere local effect of these drugs on the microbiota structure and function, our findings support the notion that a bidirectional and complex microbiota-gut-brain axis communication plays a role in addiction (Skosnik and Cortes-Briones 2016; Temko et al. 2017).

We observed that the microbiota of cocaine-treated rats was significantly less diverse compared to the control group. To some extent, these results oppose to those reported by Ning and colleagues (Ning et al. 2017) who found that the fecal microbial diversity in rats intraperitoneally treated with methamphetamine was somewhat greater than that in the control group. According to Ning and colleagues (Ning et al. 2017), this result was unexpected, since a greater bacterial diversity is conceived to be beneficial to human health, but the role of bacterial diversity in the central nervous system (CNS) function remains to be explored (Ning et al. 2017). Differences in the drugs of abuse and in the routes of administration that were used may account for the differences observed in both studies.

Caffeine is one of the most used psychoactive substances worldwide (Ferré 2016). Also, it is an adulterant frequently found

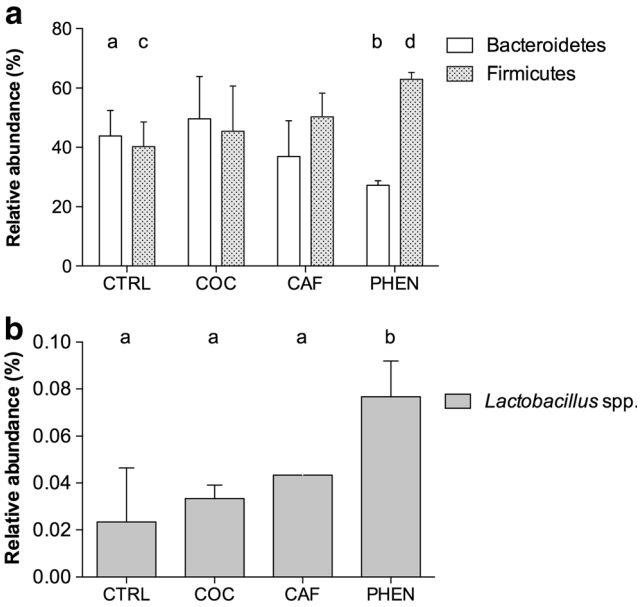

Fig. 3 Relative abundance of selected bacterial groups found in the gut of the treated rats. **a** The Firmicutes/Bacteroidetes ratio and **b** the relative abundance of *Lactobacillus* spp. Different letters indicate significant differences among treatments relative to the control treatment ($p < 0.05$; ANOVA, Bonferroni corrected). CTRL, control; COC, cocaine; CAF, caffeine; PHEN, phenacetin

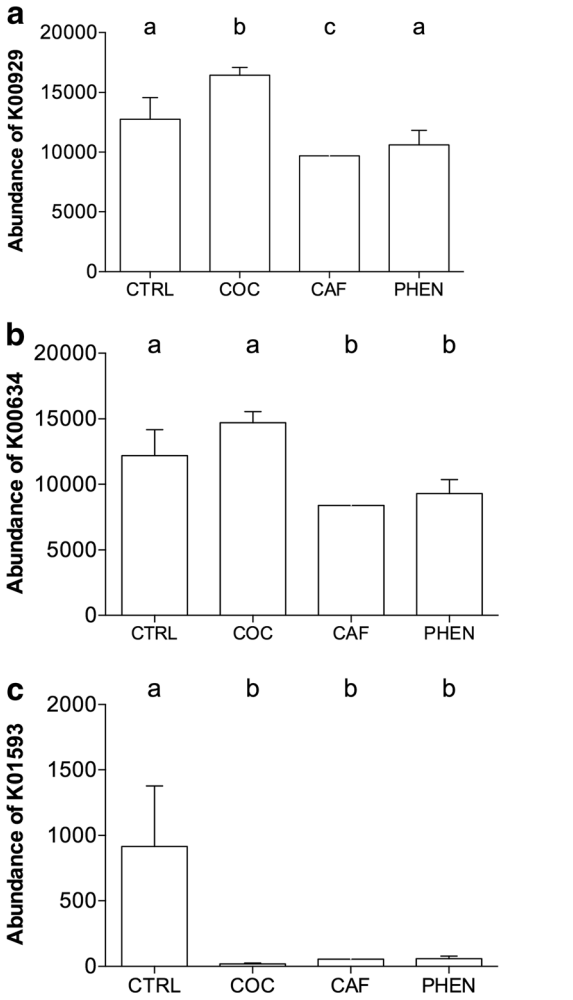

Fig. 4 Functional prediction from the obtained OTUs using PICRUSt (http://picrust.github.com). The predicted copy number of genes corresponding to butyrate kinase (**a**), phosphate butyryl transferase (**b**), and aromatic L-amino acid decarboxylase (**c**) is shown. Different letters indicate significant differences between treatments relative to the control treatment ($p < 0.05$; ANOVA, Bonferroni corrected). CTRL, control; COC, cocaine; CAF, caffeine; PHEN, phenacetin

in illicit drugs of abuse, including cocaine seized samples (Abin-Carriquiry et al. 2018; Cole et al. 2011). Moreover, it has been demonstrated that caffeine can potentiate the stimulant, the motivational and the reinforcing effects of cocaine (Broseus et al. 2016; Muñiz et al. 2017; Prieto et al. 2015, 2016). In the present study, it was observed that the fecal microbiota of caffeine-treated and control rats were similar. It seems that the diversity and structure of the gut microbiota is not sensitive to caffeine, at least at the dose and the administration route used in this study. However, phenacetin exerted notorious effects on the composition of fecal microbiota of the treated rats. Phenacetin had been extensively used as an analgesic and antipyretic drug, but due to its toxicity, it has been withdrawn from the pharmaceutical market and is now banned in several countries (Villar Núñez et al. 2018). Nonetheless, and even the particular concern about its toxicity, phenacetin is commonly used to dilute cocaine hydrochloride (Broseus et al. 2016) and also smokable forms of cocaine (Abin-Carriquiry et al. 2018).

When we analyzed the beta diversity at the OTU level, we found that fecal microbiota of cocaine- and phenacetin-treated rats were clearly different compared to those from control and caffeine treatments. The bacterial species accounting for these differences varied according to the treatment, with some OTUs specifically enriched in cocaine-treated and others in phenacetin-treated rats. Among the species enriched in both treatments compared to the control group, members of Lachnospiraceae and Ruminococcaceae have been previously described as involved in propionate and butyrate synthesis in the human gut (Reichardt et al. 2014). Interestingly, genomes of Lachnospiraceae associated to the human gut harbored genes of the butyric acid biosynthesis pathway that were not present in other non-gut associated members of this family, suggesting a host-specific ecological adaptation (Meehan and Beiko 2014). Moreover, genomes of *Lachnoclostridium*, a genus belonging to this family, show several genes for fatty acid metabolism (GenBank assembly accession GCA_001688665.2; Li et al. 2015). The specific enrichment of these bacterial groups in cocaine- and phenacetin-treated rats would imply that the functions they carry out are relevant in those environmental contexts, probably modulating the intestinal cells in drug-induced selective conditions.

When the Firmicutes/Bacteroidetes ratio was analyzed as a marker of microbiota implicated in predisposition to disease states, a significant increase was observed in the fecal microbiota of phenacetin-treated rats. Interestingly, the imbalance in the

relative proportion of Bacteroidetes and Firmicutes, with the latter prevailing, has been frequently associated to different disorders, particularly obesity (Roselli et al. 2017). We also observed that the relative abundance of lactobacilli, included in the Firmicutes phylum, significantly increased in phenacetin-treated rats compared to the other groups. This change will have to be addressed in the wider context of the changes in the microbiota of individuals that consume this substance. Overall, the analysis of the fecal microbiota composition revealed that inhaled cocaine and adulterants exerted a dysbiosis-like effect in the treated rats. The microbiota modulation or restoration using different strategies (e.g., probiotics) could be an interesting target for addiction therapy.

A bioinformatics prediction using the PICRUSt-based approach of the metagenome functional content of the fecal microbiota from the different groups of animals was performed. This bioinformatics tool allows inferring the functional pattern of microbial communities considering different genes surveyed along different samples. In our study, we observed significant changes in the metagenome content of genes related to the butyrate, dopamine, and serotonin biosynthesis. The predicted content of butyrate quinase and phosphate butyryl transferase in the gut microbiota significantly increased in cocaine-treated rats but decreased in phenacetin-treated animals. It has been reported that butyrate exerts different effects on the host playing a relevant role in brain health in several neurological disorders (Bourassa et al. 2016). Our results suggest that butyrate could be considered as a key molecule in the functioning of gut-brain axis in drug addiction.

A striking result was observed in the case of the aromatic L-amino acid decarboxylase since its predicted content significantly decreased in all the treated groups compared to the control animals. Aromatic L-amino acid decarboxylase (also known as DOPA-decarboxylase and tryptophan decarboxylase) participates in the biosynthesis of dopamine and serotonin since it catalyzes the conversion of L-DOPA to dopamine and of 5-hydroxytryptophan to serotonin (Christenson et al. 1972). Interestingly, it was proposed that aromatic L-amino acid decarboxylase could be a plausible candidate in the etiology of major psychiatric and drug abuse disorders (Ma et al. 2005). Several studies have shown that acute administration of cocaine and caffeine elicits an increase in dopamine neurotransmission in the CNS although by using different mechanism of action (Brown et al. 2001; Di Chiara and Imperato 1988; Ferré 2016). Thus, the reduction of the predicted content in L-amino acid decarboxylase induced by a chronic exposition of both drugs could be due to a compensatory change in response to a continued stimulation of the dopaminergic neurotransmission, even in the gut microbiota. Although our study did not provide an analysis of the behavioral effects induced by the chronic administration of volatilized drugs, it may be possible that the aromatic L-amino acid decarboxylase is connected to the anhedonic motivational state seen in drug users after chronic consumption of drugs (Kish et al. 2001; Volkow et al. 1992; Volkow et al. 2009). Serotonergic neurotransmission has been classically implicated in stress, mood disorders, and impulsivity, being very relevant traits in drug addiction (Arango et al. 2002). Changes in the aromatic L-amino acid decarboxylase suggest an impaired function of the brain serotonergic system elicited by chronic volatilized drugs and putative emotional alterations.

This study should be seen in light of its strengths and limitations. This is one of the few studies that has investigated the effects of cocaine exposure to the gut microbiota. Of particular bench-to-bedside translational relevance, this study was not limited to the analysis of cocaine, but it also included the analysis of two adulterants that are commonly mixed with cocaine hydrochloride or cocaine freebase. Furthermore, our route of administration is also relevant from a translational standpoint, as it is a common route used in humans and associated with increased risk to develop addiction.

Our study has some limitations, including the lack of baseline analysis of the gut microbiota, a behavioral correlation using an addiction paradigm, and whether the changes here observed may be due to local or central effects or both. These limitations will need to be addressed by future studies. Furthermore, while our approach is of potential clinical relevance (i.e., we tested not only cocaine but also adulterants added to the cocaine used by humans), experiments with larger number of animals are needed to test the potential additive or synergistic effects of these drugs on the gut microbiota. Notwithstanding these limitations, the present study provides novel information on the effects of cocaine on the gut microbiota and shows that adulterants added to cocaine (especially phenacetin) also significantly affect the gut microbiota. These results support further work to shed light the potential contribution of the gut microbiota to addictive disorders like cocaine use disorder. Furthermore, this study also points out the importance of keeping in mind the differences between drugs of abuse tested in an animal laboratory versus when the drug used in the streets by humans, where the drug of abuse may be mixed with other substances.

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Conflict of Interest The authors declare that they have no conflicts of interest.

Compliance with Ethical Standards

The study was carried out in accordance with the Instituto de Investigaciones Biológicas Clemente Estable (IIBCE) Bioethics Committee's requirements, consistent with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978), and under the current ethical regulations of the national law on animal experimentation no. 18.611.

References

Abin-Carriquiry JA, Martínez Busi M, Galvalisi M, Minteguiaga M, Prieto JP, Scorza C (2018) Identification and quantification of cocaine and active adulterants in coca-paste seized samples: useful scientific support to health care. Neurotox Res. https://doi.org/10.1007/s12640-018-9887-1

Arango V, Underwood MD, Mann JJ (2002) Serotonin brain circuits involved in major depression and suicide. Prog Brain Res 136: 443–453

Bourassa MW, Alim I, Bultman SJ, Ratan RR (2016) Butyrate, neuroepigenetics and the gut microbiome: can a high fiber diet improve brain health? Neurosci Lett 625:56–63

Broseus J, Gentile N, Esseiva P (2016) The cutting of cocaine and heroin: a critical review. Forensic Sci Int 262:73–83

Brown JM, Hanson GR, Fleckenstein AE (2001) Regulation of the vesicular monoamine transporter-2: a novel mechanism for cocaine and other psychostimulants. J Pharmacol Exp Ther 296:762–767

Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M (2016) Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. Cell 165: 1762–1775

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336

Christenson JG, Dairman W, Udenfriend S (1972) On the identity of DOPA decarboxylase and 5-hydroxytryptophan decarboxylase (immunological titration-aromatic L-amino acid decarboxylase-serotonin-dopamine-norepinephrine). Proc Natl Acad Sci U S A 69:343–347

Chung CS, Chang PF, Liao CH, Lee TH, Chen Y, Lee YC, Wu MS, Wang HP, Ni YH (2016) Differences of microbiota in small bowel and faeces between irritable bowel syndrome patients and healthy subjects. Scand J Gastroenterol 51:410–419

Clarke K (1993) Nonparametric multivariate analyses of changes in community structure. Aust J Ecol 18:117–143

Cole C, Jones L, Mcveigh J, Kicman A, Syed Q, Bellis M (2011) Adulterants in illicit drugs: a review of empirical evidence. Drug Test Anal 3:89–96

Crumeyrolle-Arias M, Jaglin M, Bruneau A, Vancassel S, Cardona A, Daugé V, Naudon L, Rabot S (2014) Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. Psychoneuroendocrinology 42:207–217

Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13: 701–712

Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A 85:5274–5278

Dinan TG, Cryan JF (2017) Gut instincts: microbiota as a key regulator of brain development, ageing and neurodegeneration. J Physiol 595: 489–503

Ferré S (2016) Mechanisms of the psychostimulant effects of caffeine: implications for substance use disorders. Psychopharmacology 233: 1963–1979

Galvalisi G, Prieto JP, Martínez M, Abin-Carriquiry JA, Scorza C (2015) Smoked cocaine: chemical analysis of seized samples and the role of caffeine in its central actions. IBRO 9th World Congress, Rio de Janeiro, Brazil. http://ibro.info/events/meetings/

Galvalisi M, Prieto JP, Martínez M, Abin-Carriquiry JA, Scorza C (2017) Caffeine induces a stimulant effect and increases dopamine release in the nucleus accumbens shell through the pulmonary inhalation route of administration in rats. Neurotox Res 31:90–98

Gossop M, Griffiths P, Powis B, Strang J (1992) Severity of dependence and route of administration of heroin, cocaine and amphetamines. Br J Addict 87:1527–1536

Grenham S, Clarke G, Cryan J, Dinan TG (2011) Brain-gut-microbe communication in health and disease. Front Physiol 2:94

Hammer Ø, Haper DAT, Ryan PD (2001) PAST: Paleontological Statistics software package for education and data analysis. Paleontol Electron 4:4–9

Hatsukami D, Fischman M (1996) Crack cocaine and cocaine hydrochloride. Are the differences myth or reality? J Am Med Assoc 276: 1580–1588

Kim BS, Jeon YS, Chun J (2013) Current status and future promise of the human microbiome. Pediatr Gastroenterol Hepatol Nutr 16:71–79

Kiraly DD, Walker DM, Calipari ES, Labonte B, Issler O, Pena CJ, Ribeiro EA, Russo SJ, Nestler EJ (2016) Alterations of the host microbiome affect behavioral responses to cocaine. Sci Rep 6:35455

Kish SJ, Kalasinsky KS, Derkach P, Schmunk GA, Guttman M, Ang L, Adams V, Furukawa Y, Haycock JW (2001) Striatal dopaminergic and serotonergic markers in human heroin users. Neuropsychopharmacology 24:561–567

Kuczynski J, Stombaugh J, Walters WA, González A, Caporaso JG, Knight R (2011) Using QIIME to analyze 16S rRNA gene sequences from microbial communities. Curr Protoc Bioinformatics Chapter 10:Unit 10.7. https://doi.org/10.1002/0471250953.bi1007s36

Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, Huttenhower C (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31:814–821

Laukens D, Brinkman BM, Raes J, De Vos M, Vandenabeele P (2016) Heterogeneity of the gut microbiome in mice: guidelines for optimizing experimental design. FEMS Microbiol Rev 40:117–132

Li H, Limenitakis JP, Fuhrer T, Geuking MB, Lawson MA, Wyss M, Brugiroux S, Keller I, Macpherson JA, Rupp S, Stolp B, Stein JV, Stecher B, Sauer U, McCoy KD, Macpherson AJ (2015) The outer mucus layer hosts a distinct intestinal microbial niche. Nat Commun 6:8292

López Hill X, Prieto J, Meikle M, Urbanavicius J, Abin-Carriquiry A, Prunell G, Umpiérrez E, Scorza C (2011) Coca-paste seized samples characterization: chemical analysis, stimulating effect in rats and relevance of caffeine as a major adulterant. Behav Brain Res 221: 134–141

Lozupone C, Hamady M, Knight R (2006) UniFrac - an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics 7:371

Ma JZ, Beuten J, Payne TJ, Dupont RT, Elston RC, Li MD (2005) Haplotype analysis indicates an association between the DOPA decarboxylase (DDC) gene and nicotine dependence. Hum Mol Genet 14:1691–1698

Mathur R, Barlow GM (2015) Obesity and the microbiome. Expert Rev Gastroenterol Hepatol 9:1087–1099

Meehan CJ, Beiko RG (2014) A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. Genome Biol Evol 6:703–713

Muñiz JA, Prieto JP, González B, Sosa MH, Cadet JL, Scorza C, Urbano FJ, Bisagno V (2017) Cocaine and caffeine effects on the conditioned place preference test: concomitant changes on early genes within the mouse prefrontal cortex and nucleus accumbens. Front Behav Neurosci 11:200

Ning T, Gong X, Xie L, Ma B (2017) Gut microbiota analysis in rats with methamphetamine-induced conditioned place preference. Front Microbiol 8:1620

Pérez-Martínez G, Bäuerl C, Collado MC (2014) Understanding gut microbiota in elderly's health will enable intervention through probiotics. Benef Microbes 5:235–246

Prieto JP, Galvalisi M, López Hill X, Meikle MN, Abin-Carriquiry JA, Scorza C (2015) Caffeine enhances and accelerates the expression of sensitization induced by coca paste indicating its relevance as a main adulterant. Am J Addict 24:475–481

Prieto JP, Scorza C, Serra GP, Perra V, Piras G, Galvalisi M, Abin-Carriquiry JA, Valentini V (2016) Cocaine motivational value is enhanced when co-administered with caffeine: relevance of adulterants in reinforcement. Psychopharmacology 233:2879–2889

Reichardt N, Duncan SH, Young P, Belenguer A, McWilliam Leitch C, Scott KP, Flint HJ, Louis P (2014) Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. ISME J 8:1323–1335

Rieder R, Wisniewski PJ, Alderman BL, Campbell SC (2017) Microbes and mental health: a review. Brain Behav Immun 66:9–17

Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. PeerJ. 4:e2584

Roselli M, Devirgiliis C, Zinno P, Guantario B, Finamore A, Rami R, Perozzi G (2017) Impact of supplementation with a food-derived microbial community on obesity-associated inflammation and gut microbiota composition. Genes Nutr 4:12–25

Samaha AN, Robinson TE (2005) Why does the rapid delivery of drugs to the brain promote addiction? Trends Pharmacol Sci 26:82–87

Sekirov I, Finlay BB (2009) The role of the intestinal microbiota in enteric infection. J Physiol 587:4159–4167

Skosnik PD, Cortes-Briones JA (2016) Targeting the ecology within: the role of the gut-brain axis and human microbiota in drug addiction. Med Hypotheses 93:77–80

Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J (2017) New evidences on the altered gut microbiota in autism spectrum disorders. Microbiome 5:24

Temko JE, Bouhlal S, Farokhnia M, Lee MR, Cryan JF, 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

Villar Núñez MLÁ, Sánchez Morcillo J, Ruíz Martínez MA (2018) Purity and adulteration in cocaine seizures and drug market inspection in Galicia (Spain) across an eight-year period. Drug Test Anal 10:381–391

Volkow ND, Hitzemann R, Wang GJ, Fowler JS, Wolf AP, Dewey SL, Handlesman L (1992) Long-term frontal brain metabolic changes in cocaine abusers. Synapse 11:184–190

Volkow ND, Fowler JS, Wang GJ, Baler R, Telang F (2009) Imaging dopamine's role in drug abuse and addiction. Neuropharmacology 56:3–8

Volpe GE, Ward H, Mwamburi M, Dinh D, Bhalchandra S, Wanke C, Kane AV (2014) Associations of cocaine use and HIV infection with the intestinal microbiota, microbial translocation, and inflammation. J Stud Alcohol Drugs 75:347–357

Wiley NC, Dinan TG, Ross RP, Stanton C, Clarke G, Cryan JF (2017) The microbiota-gut-brain axis as a key regulator of neural function and the stress response: implications for human and animal health. J Anim Sci 95:3225–3246

Williams BB, Van Benschoten AH, Cimermancic P, Donia MS, Zimmermann M, Taketani M et al (2014) Discovery and characterization of gut microbiota decarboxylases that can produce the neurotransmitter tryptamine. Cell Host Microbe 16:495–503

Xiao HW, Ge C, Feng GX, Li Y, Luo D, Dong JL, Li H, Wang H, Cui M, Fan SJ (2018) Gut microbiota modulates alcohol withdrawal-induced anxiety in mice. Toxicol Lett 287:23–30

Zhang Q, You J, Volkow ND, Choi J, Yin W, Wang W, Pan Y, Du C (2016) Chronic cocaine disrupts neurovascular networks and cerebral function: optical imaging studies in rodents. J Biomed Opt 21: 26006

Zhu YY, Li H, Xu XL, Li CB, Zhou GH (2016) The gut microbiota in young and middle-aged rats showed different responses to chicken protein in their diet. BMC Microbiol 16:281