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Short communication

Drunk bugs: Chronic vapour alcohol exposure induces marked changes in the gut microbiome in mice

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HIGHLIGHTS

- Mice chronically exposed to ethanol vapours exhibited marked changes in microbiota.
- Ethanol exposure significantly increases in genus Alistipes.
- Ethanol exposure reduces many bacterial taxa, most significantly Clostridium.
- Chronic ethanol exposure caused reductions in bacteria alpha diversity.
- Bacterial changes found align with previous findings associated to inflammation.

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ABSTRACT

The gut microbiota includes a community of bacteria that play an integral part in host health and biological processes. Pronounced and repeated findings have linked gut microbiome to stress, anxiety, and depression. Currently, however, there remains only a limited set of studies focusing on microbiota change in substance abuse, including alcohol use disorder. To date, no studies have investigated the impact of vapour alcohol administration on the gut microbiome. For research on gut microbiota and addiction to proceed, an understanding of how route of drug administration affects gut microbiota must first be established. Animal models of alcohol abuse have proven valuable for elucidating the biological processes involved in addiction and alcohol-related diseases. This is the first study to investigate the effect of vapour route of ethanol administration on gut microbiota in mice. Adult male C57BL/6J mice were exposed to 4 weeks of chronic intermittent vapourized ethanol (CIE, N = 10) or air (Control, N = 9). Faecal samples were collected at the end of exposure followed by 16S sequencing and bioinformatic analysis. Robust separation between CIE and Control was seen in the microbiome, as assessed by alpha ($p < 0.05$) and beta ($p < 0.001$) diversity, with a notable decrease in alpha diversity in CIE. These results demonstrate that CIE exposure markedly alters the gut microbiota in mice. Significant increases in genus *Alistipes* ($p < 0.001$) and significant reductions in genra *Clostridium* IV and XIVb ($p < 0.001$), *Dorea* ($p < 0.01$), and *Coprococcus* ($p < 0.01$) were seen between CIE mice and Control. These findings support the viability of the CIE method for studies investigating the microbiota-gut-brain axis and align with previous research showing similar microbiota alterations in inflammatory states during alcoholic hepatitis and psychological stress.

The gut microbiota includes a community of bacteria that play an integral part in nutrient metabolism and absorption in addition to gating host immune function [1]. Recently, a growing body of evidence points to the presence of a microbiota-gut-brain axis. Indeed, preclinical studies have associated commensal bacteria to hypothalamic–pituitary–adrenal (HPA) signalling [1], neurodevelopment processes [2], such as myelination [3], in addition to various

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behavioural phenotypes [1]. Currently, however, there remains only a limited set of studies focusing on microbiota change in substance abuse, including alcohol use disorder [4–6]. To date, no studies have investigated the impact of vapour alcohol administration on the gut microbiome.

Chronic alcohol abuse can cause damage to health including nutrient depletion, cognitive deficits, and alcoholic liver disease. Animal models of alcohol abuse have proven valuable for elucidating the biological processes involved in addiction, fetal alcohol syndrome, and alcohol-related diseases. A widely used murine model of chronic alcohol abuse is chronic intermittent ethanol (EtOH) (CIE) exposure because it resembles the prolonged, repeated patterns of alcohol abuse seen in humans [7,8]. Prior studies using the CIE model have reported changes in a range of neural indices and behavioural phenotypes, including increased EtOH self-administration [9–13].

The CIE model could be advantageous for studying potential changes in the gut microbiome resulting from chronic EtOH exposure, as compared to other methods such as EtOH drinking, because (1) there is less potential for confounding effects of caloric intake (2) administration dose is consistent among subjects, and (3) vapour administration circumvents the physiological effects of EtOH in the gastrointestinal tract, such as altered nutrient absorption. For these reasons, the current study employed the CIE method in order to investigate the consequences of chronic vapour EtOH on gut microbiome.

Adult male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) at 7 weeks of age. Mice were housed two per cage in a temperature (72 ± 5 °F) and humidity (45 ± 15%) controlled SPF vivarium on a 12 h light/dark cycle (lights on 06:00). Animals were allowed to acclimate to the facility for 1 week prior to CIE. Food and water was provided ad libitum for the duration of the experiment. Food, NIH-31 Harlan/Teklad Roden Chow, was obtained from Harlan Teklad (Indianapolis, IN, USA). Cages were changed the same day each week (Mondays) and fresh Teklad corn cob bedding (1/8") was also obtained from Harlan Teklad. Experimental procedures were approved by the NIAAA Animal Care and Use Committee and followed NIH guidelines.

Subjects were randomly assigned to either the air (Control) or CIE group; N = 10 for each group. A previously described vapour inhalation procedure was employed [8,11]. Prior to being placed in the vapour chambers, test subjects received IP injections of 1.5 g/kg of 20% EtOH (v/v) with 71.6 mg/kg of an alcohol dehydrogenase (ADH) inhibitor, pyrazole (Sigma, St. Louis, MO, USA), in a combined volume of 10 mL/kg body weight, to initiate intoxication and stabilize blood EtOH concentrations (BECs). The average weight of mice was ~22 g for both groups. During exposure, mice were removed from their home cages and singly housed in clean 60 × 36 × 60 cm cages (PlasLabs, Lansing, MI, USA) and placed into Plexiglas vapour chambers. 95% EtOH was passed through a vapourization stone at 19–22 mg EtOH/L of fresh air and delivered at a rate of ~10 L/min. BECs were measured in sentinels using the Analox AM1 alcohol analyzer (Analox Instruments USA, Lunenburg, MA, USA) and achieved BECs of 175 ± 25 mg/dL. The protocol for Control group was similar to the CIE. The Control group received a 68.1 mg/ kg IP injection of pyrazole and was exposed to air at a rate of ~10 L/min in Plexiglas vapour chambers directly adjacent to the EtOH vapour chambers. Pyrazole dose was adjusted based on solubility for mice to receive equal concentrations, thus dosage was higher in the CIE treated group because the solubility of pyrazole (a hydrophobic heterocyclic compound) is lower in EtOH than in saline (0.9% NaCl). Vapour exposure occurred for 16 h per day (17:00–09:00), 5 days a week (Monday–Friday) for 4 consecutive weeks. Mice were 8 weeks of age at the beginning of the study and 12 weeks of age at the end of the study.

We utilized a previously published method for isolating microbiota from faecal samples under sterile conditions [14]. Immediately following the final exposure, mice from both groups were euthanized via cervical dislocation and caecal and colonic contents were harvested, pooled and diluted 40-fold (weight: volume) in sterile water. After centrifugation at 800 RPM, the supernatant was aliquoted under sterile conditions for storage at −80 °C.

The QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract bacterial DNA from sample dilutions. Extracted DNA was further concentrated using a standard EtOH precipitation protocol. The prokaryotic 16S ribosomal RNA gene (16S rRNA) was amplified from extracted DNA using amplicon PCR for the V3 and V4 regions following the *Illumina 16S Sample Preparation Guide*. Using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), 16S rRNA amplicons were further prepared for 2 × 250 bp sequencing on the Illumina MiSeq platform. The Illumina V3–V4 primers were selected for this study because they have a high coverage (94.5% bacteria) while remaining in the amplicon size necessary to sequence at 2 × 250 bp [15].

All sequences in FASTQ files format were filtered using PRINSEQ. Sequences with length less than 150 nucleotides or with low quality at the 3′ end were removed. Paired-end reads with a minimum overlap of 20 base-pairs were joined using FASTQ-join. Finally, all single files were processed to a final filtering sequence (mean quality score >20). Sequences from one sample in the Control group had to be removed due to low sequencing reads, which resulted in a low quality score (<20). After filtering quality and length trimming, the average number of high-quality sequences generated per sample (Control N = 9, CIE N = 10) was 63,600 ± 25,932 SD. The average number of OTUs per sample was 649 ± 128 SD.

The sequences were matched at operational taxonomic unit (OTU; 97% identity level) using closed-reference USEARCH v7.0 algorithm against The Ribosomal Database Project. Alpha and beta diversity was determined using QIIME. Additional alpha and beta diversity analyses were performed with the R package phyloseq.

Alpha diversity was computed based on Shannon and Simpson methods and was visualized via the phyloseq package. Family and genera-level analyses were carried out using the Kruskal–Wallis method with the phyloseq package. Adjusted p-values (q-value) correct for multiple testing according to the method of Benjamini and Hochberg [16] and Bonferroni. Post hoc analysis of statistically significant taxa was performed to calculate log2 fold change in CIE relative to Control, p-values were estimated using negative binomial distribution (Gamma-Poisson).

The Shannon and Simpson indexes are the most commonly used formulas for calculating the alpha diversity of microbiota [17]. These indexes showed significant (Shannon and Simpson indexes, $p < 0.05$) differences in alpha diversity, with mean diversity decreased in the CIE group, relative to controls (Fig. 1A).

Sample distribution by sequenced OTUs using Principle Coordinate Analysis (PCoA) revealed phylogenetic separation between CIE and Control groups (Fig. 1B). Beta diversity analysis comparing taxonomic similarities between individual samples was calculated with Bray-Curtis (Fig. 1C). The heatmap shows correlations of taxonomic OTU composition for each sample compared to every other sample in the study. Samples are distributed by overall taxonomic OTU similarity, regardless of group designation. This method is used to assess if subjects cluster by exposure method, similar to a PCoA plot. In the Bray-Curtis heatmap, subjects clustered by exposure group (Fig. 1C). Multivariate analysis of beta diversity between CIE and Control groups revealed significant differences in total sequenced OTUs (ANOSIM and Adonis, $p < 0.001$) and at the genus level (ANOSIM, $p < 0.01$) but no significant difference at the family level (ANOSIM, $p = 0.157$).

At the family level, data revealed a significant difference in the Rikenellaceae family (Kruskal–Wallis, $p < 0.001$) between