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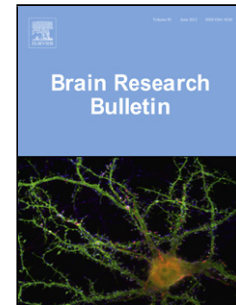
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**Drunk Bugs: Chronic Vapour Alcohol Exposure Induces Marked Changes
in the Gut Microbiome in Mice**

Category submitted: Short Communication – Behavioural Brain Research

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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

The gut microbiota includes a community of bacteria that and 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 (Shannon and Simpson index, $p < 0.05$) and beta (ANOSIM, $p < 0.001$) 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 genera *Clostridium* IV and XIVb (Kruskal-Wallis, $p < 0.001$), *Dorea* (Kruskal-Wallis, $p < 0.01$), and *Coprococcus* (Kruskal-Wallis, $p < 0.01$) 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.

KEYWORDS: Chronic alcohol, microbiome, vapour ethanol, microbiota-gut-brain axis

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 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 ($72\pm 5^{\circ}\text{F}$) and humidity ($45\pm 15\%$) controlled SPF vivarium on a 12 hour 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 grams for both groups. During exposure, mice were removed from their home cages and singly housed in clean $60\times 36\times 60$ 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 ~10L/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 hours 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 fecal samples under sterile conditions [14]. Immediately following the final exposure, mice from both groups were euthanatized via cervical dislocation and cecal 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 samples 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 2x250bp 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 2x250bp [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 $63,600 \pm 25,932$ 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. Corrected p-values (q-value) adjust for multiple testing according to the method of Benjamini and Hochberg [16]. 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 (Figure 1A).

Sample distribution by sequenced OTUs using Principle Coordinate Analysis (PCoA) revealed phylogenetic separation between CIE and Control groups (Figure 1B). Beta diversity analysis comparing taxonomic similarities between individual samples was calculated with Bray-Curtis (Figure 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 (Figure 1C). Multivariate analysis of beta diversity between CIE and Control groups revealed significant differences in total sequenced OTUs (ANOSIM, $p < 0.001$ or 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 groups, which remained statistically significant when corrected for multiple comparisons (Bonferonni, $q\text{-value} < 0.05$) (Figure 2B). Rikenellaceae was significantly increased (\log_2 fold change > 3.5 , $p < 0.0001$) in CIE relative to Control

(Figure 2B).

At the genus level, data revealed significant differences in *Alistipes* (Kruskal-Wallis, $p < 0.001$) and *Clostridium* IV and XIVb (Kruskal-Wallis, $p < 0.001$) between CIE mice and Control, which remained statistically significant when corrected for multiple comparisons (Bonferonni, $q\text{-value} < 0.05$) (Figure 2B). A higher abundance of *Alistipes* (\log_2 fold change > 3.8 , $p < 0.0001$) was seen in the CIE group, with decreases in *Clostridium* IV (\log_2 fold change > -1.4 , $p < 0.001$) and *Clostridium* XIVb (\log_2 fold change > -2.0 , $p < 0.001$) (Figure 2).

Less statistically significant changes were seen for *Dorea* (Kruskal-Wallis, $p < 0.01$), *Coprococcus* (Kruskal-Wallis, $p < 0.01$), and *Propionibacterium* (Kruskal-Wallis, $p < 0.01$), which did not remain statistically significant when corrected for multiple comparisons (Bonferonni, $q\text{-value} = 0.23$) but did remain significant when corrected for false discovery rate (FDR, $q\text{-value} < 0.05$). *Akkermansia* was visibly increased in the CIE graph of bacterial composition (Figure 2A), but significance (Kruskal-Wallis, $p < 0.05$) did not hold after corrections (Bonferonni $q\text{-value} = 1.00$). Significant decreases in *Dorea* (\log_2 fold change > -2.5 , $p < 0.01$), *Coprococcus* (\log_2 fold change > -1.6 , $p < 0.05$), and *Propionibacterium* (\log_2 fold change > -1.7 , $p < 0.05$) were observed in the CIE group relative to Control (Figure 2).

Although disturbances in the microbiome are linked to a variety of health states and the action of a variety of pharmacological agents, there is a paucity of information on the impact of chronic EtOH on microbiome composition [18]. Here we found that the bacterial composition in mice exposed to CIE, as measured from Bray-Curtis and PCoA analyses, exhibited a clear separation from that seen in air-exposed Controls. These data add to a growing literature showing how changes in the microbiome can occur independently of direct administration of a substance into the gut [19]. They also extend the findings of a previous report showing reductions in alpha diversity following chronic alcohol feeding [4]. Taken together with the current findings, these results demonstrate alterations in the gut microbiota in response to chronic EtOH across a range of routes of administration.

In this study, *Alistipes* was the only genus of bacteria that significantly increased with CIE. Increases in *Alistipes* and reductions in *Clostridium cluster IV* have been found in mice receiving fecal microbiota transfer from alcoholic patients with severe hepatitis [5]. An increased population of *Alistipes* has also been observed in chronic fatigue syndrome, inflammation, inflammatory bowel syndrome, and depression [20, 21]. We observed a decrease in genus *Dorea* in CIE relative to Control (Figure 2B). Conversely, a study by Leclercq and colleagues found increases in *Dorea* associated with high intestinal permeability in alcoholics. However, our current study found reductions in *Clostridium cluster IV* also seen in this aforementioned study [6]. The genus *Clostridium cluster IV* is populated by bacterial species believed to confer an anti-inflammatory effect and therefore beneficial to the host [5]. *Faecalibacterium prausnitzii* and *Clostridium leptum* are among these beneficial species within this genus and are reduced in studies of alcohol

consumption and hepatitis [5, 6]. There is limited information about the functions of genera *Propionibacterium* and *Clostridium* XIVb within the gut microbiome though they are known to include species that produce short-chain fatty acids [22, 23] which are implicated in behavior and cognition [24]. Similar reductions in *Dorea* and *Coprococcus* which have previously been observed in chronic social stress and correlated to increases in pro-inflammatory cytokines IL-6 and MCP-1 [19]. Future studies are needed to examine the relative importance of these alterations to the pathophysiology in disease states.

Despite its advantage of mimicking the most common route of alcohol administration in humans, oral EtOH self-administration in rodents has several limitations, including: 1) dosage variability between test subjects, 2) inconsistent and unreliable self-administration in animals, 3) difficulty in achieving and maintaining a desired BEC, and 4) experimental confounds such as caloric intake [10]. While vapour exposure is not a common form of achieving intoxication in humans, this method is valuable in differentiating the underlying mechanisms involved in microbial changes caused by gastrointestinal EtOH exposure and other changes acting through the brain-gut-microbiota axis. A limitation in using this CIE model of vapour exposure is the administration of pyrazole, a known hepatotoxin, which may confound result by having a synergistic effect with ethanol [25] that may further alter liver function, inflammation, and gut microbiome. No overt signs of liver damage were observed in either the AIR or CIE mice during the necropsies that immediately followed the respective exposures. It is possible that by using the V3-V4 16S amplification primers, certain bacterial taxa may have been missed and/or misrepresented, though limitations exist within every sequencing method that could potentially affect results. Finally,

differences in microbiota composition across strains [26, 27] and vendors [28] is a significant factor affecting reproducibility in animal studies. To generalize results outside of strain or environment, the functional ecology of the microbiome must be examined [29]. We acknowledge that some changes in bacteria seen in this study may not be seen in another animal strain under the same experimental conditions, however changes in other bacteria with the same functional traits are likely to be observed.

In conclusion, the current study reveals marked changes in the bacterial composition of faecal microbiota in C57BL/6J mice chronically exposed to EtOH vapours. These findings align with and extend a growing body of evidence of microbiota changes associated with inflammation and support the viability of the CIE method for future studies investigating the effects of EtOH on the microbiota-gut-brain axis. Furthermore, the robust effects of EtOH vapour suggest that other substances of abuse administered outside of the gastrointestinal system may potentially alter gut microbiota.

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The authors have no potential conflicts of interest to report.

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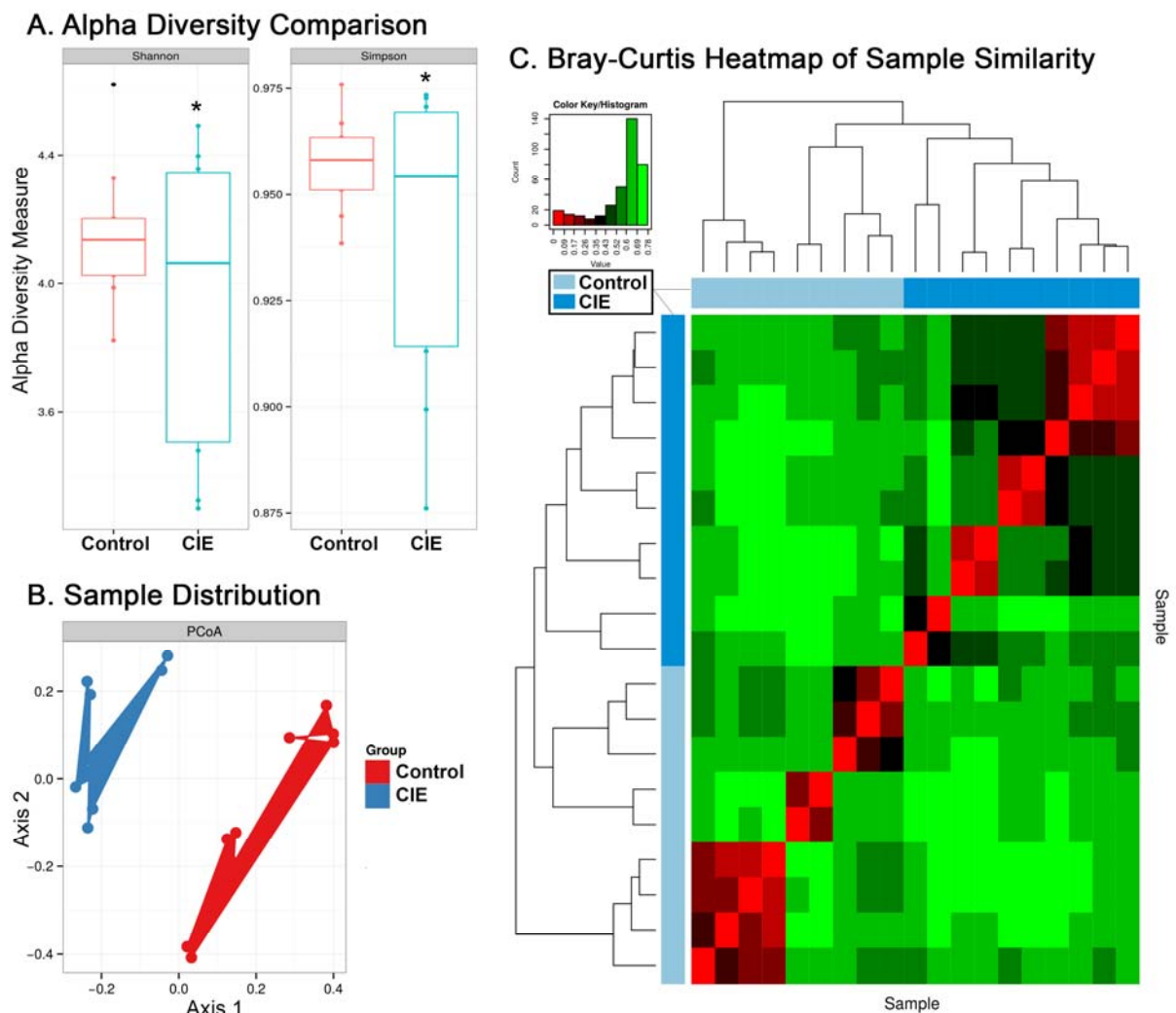


Figure 1: Effects of CIE vapour exposure on the microbiome. (A) Shannon and Simpson Index alpha diversity measures of bacterial number and distribution by group. Group outliers indicated with a dot, significance ($p < 0.05$) indicated with an asterisk. (B) PCA plot showing distribution of samples by OTUs. Clusters are colored based on their respective experimental group. (C) Bray-Curtis heatmap of beta diversity correlations between samples. Axes indicate: i) individual sample per row (right y-axis) and column (bottom x-axis) and ii) dendrogram showing taxonomic relationships by total OTUs with blue bar showing group assignment (left y-axis, top x-axis).

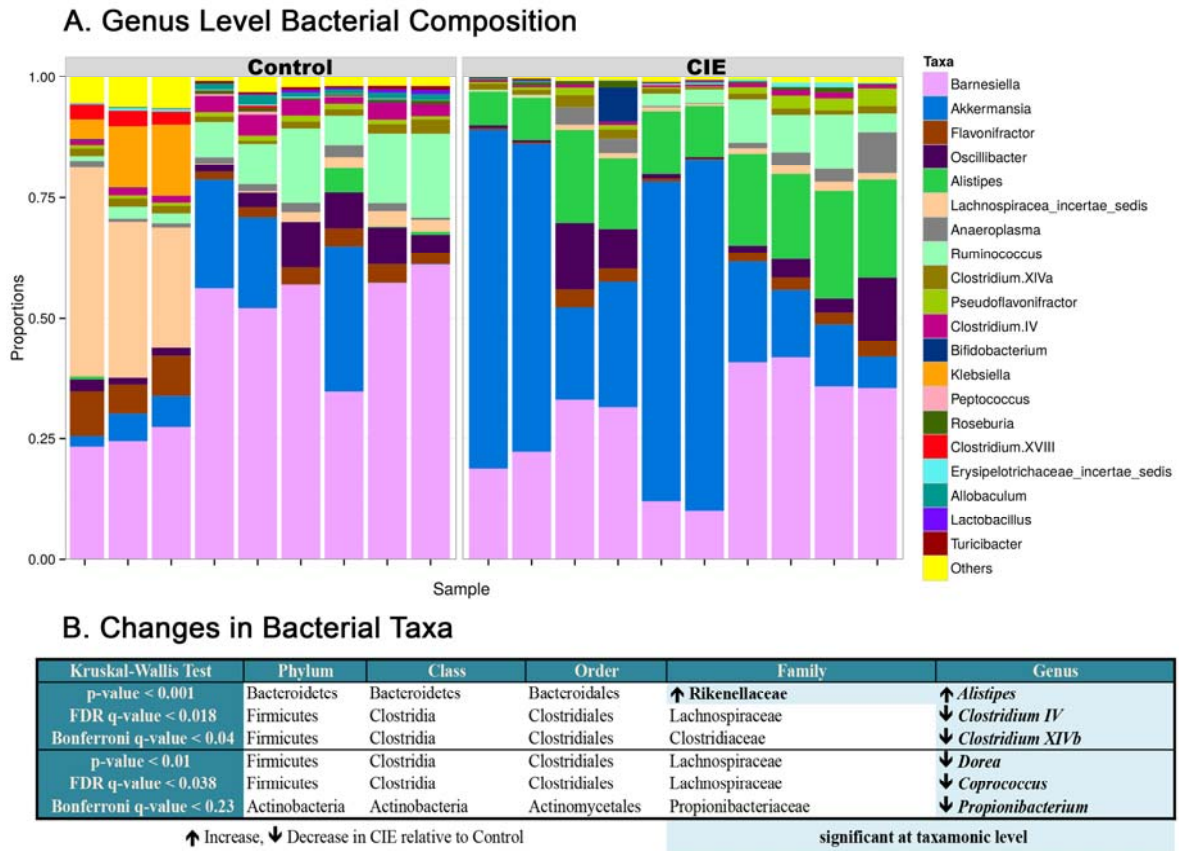


Figure 2: Effects of CIE vapour exposure on bacterial composition. (A) Genus level bacterial composition by group. (B) Statistically significant taxonomic changes in CIE exposed relative to Controls. Statistically significant taxa highlighted in blue and grouped into rows ordered based on p-value.