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Alcohol-induced changes in the gut microbiome and metabolome of rhesus macaques

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Abstract

Rationale—Increasing evidence has demonstrated that changes in the gut microbiome, including those associated with dietary influences, are associated with alterations in many physiological processes. Alcohol consumption is common across human cultures and is likely to have a major effect on the gut microbiome, but there remains a paucity of information on its effects in primates.

Objectives—The effects of chronic alcohol consumption on the primate gut microbiome and metabolome was studied in rhesus macaques that were freely drinking alcohol. The objectives of the study were to determine what changes occurred in the gut microbiome following long-term exposure to alcohol and if these changes were reversible following a period of abstinence.

Methods—Animals consuming alcohol were compared to age matched controls without access to alcohol and were studied before and after a period of abstinence. Fecal samples from rhesus macaques were used for 16S rRNA sequencing to profile the gut microbiome and for metabolomic profiling using mass spectrometry.

Results—Alcohol consumption resulted in a loss of alpha-diversity in rhesus macaques, though this was partially ameliorated by a period of abstinence. Higher levels of Firmicutes were observed in alcohol drinking animals at the expense of a number of other microbial taxa, again normalizing in part with a period of abstinence. Metabolomic changes were primarily associated with differences in glycolysis when animals were consuming alcohol and differences in fatty acids when alcohol-drinking animals became abstinent.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Conclusions—The consumption of alcohol has specific effects on the microbiome and metabolome of rhesus macaques independent of secondary influences. Many of these changes are reversed by a relatively short period of abstinence.

Keywords

alcohol; microbiome; metabolome; rhesus macaque

Introduction

Alcohol consumption is common across cultures and history, but only in the last two hundred years has society begun to associate it with negative consequences (Levine 1984). While the effects of consumption in moderate amounts remains muddled, over-consumption has been associated with a number of health-related issues (Rehm et al. 2009; Shield et al. 2013). Physiologically, it has been associated with various cancers (Corrao et al. 2004), heart disease (Taylor et al. 2009), diabetes (Baliunas et al. 2009), and more. Alcohol consumption and alcohol use disorders are also associated with major depressive disorder and anxiety disorders (Boschloo et al. 2011; Conner et al. 2009) and comorbid with a number of other psychiatric diseases although order of causation is unclear. In the past decade, the effect of alcohol on the gut microbiome has begun to garner attention as a target of both pathology and treatment (Cresci 2015; Hillemecher et al. 2018; Leclercq et al. 2018).

There have been a number of studies that have attempted to associate alcohol consumption with changes to the microbiome, the vast majority of which are correlative in nature. In humans, there has been evidence of microbiome dysbiosis associated with patients with chronic alcoholic dependence (Leclercq et al. 2014; Mutlu et al. 2012) but only with a subset of individuals and correlated neither with physiological liver disease nor period of abstinence. The individuals with greater microbiome dysbiosis also demonstrated a “leaky gut”, increased intestinal permeability and transfer of luminal biochemicals, and had more severe alcohol use disorders, depression, and anxiety (Leclercq et al. 2014). Patients with alcohol-derived liver pathologies demonstrate distinct microbiome community profiles from controls including a significantly decreased microbial diversity (Chen et al. 2011; Sarangi et al. 2017). This work has also been extended to include other alcohol-derived liver and pancreas pathologies and to compare not only these patients to healthy controls, but also to patients with alcohol use disorders absent these additional pathologies (Ciocan et al. 2018; Dubinkina et al. 2017). These latter studies demonstrated that additional distinct changes in the microbiome occur associated with alcohol-derived diseases of the liver and pancreas beyond the changes occurring from alcohol consumption alone.

Human studies of the microbiome, however, have many challenges (Kelly et al. 2016). Inter-individual variability in human populations is high and unsurprising given the vast differences in diet and environment that can be particularly pronounced on the microbiome (Lozupone et al. 2012). In studies of alcohol, this is compounded by differences in quantity, duration, and co-variable environmental exposures. These differences in human studies are highlighted by the differences seen both in groups with comorbid liver or pancreas pathologies (Ciocan et al. 2018; Dubinkina et al. 2017) or severity of alcohol use disorder

(Leclercq et al. 2014). However, while study specifics varied, a commonality was the observation of a decrease in microbiome diversity with alcohol exposure. Illuminatingly, the one exception to this decrease in diversity was observed in the citizen science American Gut Project (Kosnicki et al. 2018). It is tempting to impute sociological meaning to these differences in results, but it can suffice to demonstrate that in humans confounds in populations under study remain an important consideration.

As for much of biomedical science, the majority of foundational studies on the relationship between alcohol and the gut microbiome, particularly the gut-brain axis and behavior, are derived from rodents (Cryan and Dinan 2012; Sampson and Mazmanian 2015; Vuong et al. 2017). Some of the first studies to demonstrate gut dysbiosis resulting from alcohol were done in rats (Mutlu et al. 2009) and mice (Bull-Otterson et al. 2013; Yan et al. 2011). Since then, there have been a number of follow-up studies using rodents with varying models of alcohol administration and showing generally, but not always, similar decreases in diversity and specific shifts in microbial abundance, particularly in Firmicutes and Bacteroidetes (Jadhav et al. 2018; Kosnicki et al. 2018; Lowe et al. 2017; Peterson et al. 2017; Wang et al. 2018). It is unclear, however, how the gut microbiome of rodents can be translated to humans. The colonic contents are processed differently in rodents and the native composition of their microbiome is dissimilar (Kostic et al. 2013). Rodents also metabolize alcohol differently than humans (Cederbaum 2012) and models of alcohol consumption in rodents are often of short duration and high concentration (Bertola et al. 2013), a pattern not commonly seen in human populations.

Non-human primate models such as the rhesus macaque (*Macaca mulatta*) are widely used in biomedical research because of their behavioral, genetic, and physiological similarities to humans (Vallender and Miller 2013). The gastrointestinal tract of the rhesus macaque also is similar to humans (Dubois et al. 1977) and previous research has demonstrated that the compositional structure of the macaque microbiome is comparable to that of humans as well (Handley et al. 2012; McKenna et al. 2008; Yasuda et al. 2015). Rhesus macaque models of alcohol consumption have also been developed that better capture the behavioral patterns seen in humans (Platt and Rowlett 2012). Recently rhesus macaque mucosal biopsies from multiple sites in the intestinal tract were used to investigate the effects of long-term alcohol self-administration on the gut microbiome (Barr et al. 2018). In contrast to the majority of human and rodent studies, this research did not identify differences in diversity levels following alcohol consumption. It also showed decreases in Bacteroidetes and increases in Proteobacteria, again contrasting with previous work. These discrepancies are hypothesized to result from differences in duration and quantity of alcohol consumption, diet, and physiological differences. It is also likely that they are affected by differences between mucosal and luminal microbial communities (Yasuda et al. 2015).

The reversibility, or irreversibility, of alcohol-induced changes to the gut microbiome is important in the context of treatment. Longitudinal studies can offer not only better understandings of the causes and effects of alcohol on the microbiome, but also lay the groundwork for interventional work. The animal studies, including both the human and rhesus macaque, tend to use single time points, collecting samples post-mortem or during drinking periods. Most human studies are single time point as well, but with participants

varying in their last exposure to alcohol, though most of these studies are of chronic, high quantity alcohol drinkers that often have developed secondary liver diseases as a result. One study in humans included the effects of a 3-week abstinence period, finding few changes exception for an increase in total bacterial levels and a handful of specific genera (Leclercq et al. 2014).

Here, we use a rhesus macaque model to explore the effects of chronic alcohol self-administration on the gut microbiome and metabolome. We also test the effects of a short, 5-day, abstinence period. These studies are aimed at developing a rhesus macaque model of the human gut microbiome in disease that can be used to investigate translationally-relevant environmental, pharmacological, and interventional manipulations with the strong controls necessary. With this work we hope to improve our understanding of the relationship between alcohol consumption and microbiome changes, which may ultimately lead to the development of novel therapeutic tools for alcohol-related issues.

Materials and methods

Animals

Samples from 21 outbred male Indian-origin rhesus macaques (*Macaca mulatta*) were used in this study. At the time of study, animals were either adolescent (4–6 years; n=12) or adult (10–15 years; n=9) and weighed 7–10 kg or 10–12 kg respectively. All animals were individually housed with a 12:12 hour light/dark cycle and fed standard monkey chow (Harlan Teklad Monkey Diet, Harlan Teklad, Madison, WI). Diets were supplemented with fresh fruit but did not vary between animals. Monkeys were divided into either alcohol drinking or control groups of adolescent alcohol (n=6), adolescent control (n=6), adult alcohol (n=4) and adult control (n=5). All procedures were performed in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the National Academies Guide for Care and Use of Laboratory Animals (National Research Council 2011). All protocols were approved by the Harvard Medical School Institutional Animal Care and Use Committee.

Alcohol self-administration

For all animals, alcohol self-administration occurred in the home cage 5 days per week via a custom-designed operant drinking panel attached to one side of the cage. The panel was equipped with two response levers, two retractable sippers equipped with solenoids to minimize dripping (model #: ENV-652AM), a food pellet dispenser, and stimulus lights (Med Associates, Inc., St. Albans, VT). Sippers were attached to stainless steel liquid reservoirs fixed on the outside of the drinking panel via Tygon tubing.

Monkeys were induced to drink alcohol in a step-wise fashion similar to previous studies (Vivian et al. 2001), with each “step” lasting 30 days. Initially, only water was available from 1 spout. In this step, every lever press (fixed-ratio 1; FR1) during the 3-h session resulted in the extension of the sipper for 30-s. Depression of the sipper by the monkey resulted in fluid delivery. Within the 30-s sipper extension time, the monkey could stop drinking (i.e., release the spout) and resume drinking (i.e., displace the spout) as many times

as he wanted. The actual duration and volume of intake, within the constraints of the schedule, were under the control of the subject. Between extensions of the drinking spout, all lights were off briefly, and responses had no scheduled consequences. To facilitate use of the panel and development of drinking behavior, during water training sessions, food pellets were delivered into a receptacle every 600 s (i.e., schedule-induced polydipsia; (Falk 1961; Vivian et al. 2001)).

In the second step, the volume of a 4% w/v ethanol solution (95% ethanol diluted with tap water; Pharmco Products, Brookfield, CT) required to deliver a dose of 0.5 g/kg alcohol was made available to drink on the opposite sipper; in the third step, the volume of 4% w/v ethanol solution was increased to deliver a dose of 1 g/kg; and, finally, in the fourth step, the volume of 4% w/v ethanol solution was increased again to deliver a dose of 1.5 g/kg. Other aspects of the self-administration procedure (e.g., sipper extension time, fixed-ratio, session length, scheduled delivery of pellets) were the same as during water only availability (step 1). Following induction, the adult animals were given concurrent access to both water and ethanol (0.5 – 16% w/v depending on phase of study) and could press either lever to obtain the fluid of their choice. Pellet delivery also was discontinued.

All adolescent animals self-administered alcohol for a period of 3 months (i.e., step-wise induction), while the adult animals had been allowed to self-administer alcohol for 5 years with irregular breaks as necessary for standard animal husbandry. All animals reliably self-administered alcohol across the period of study (Sawyer et al. 2014). Average daily alcohol consumption in the adolescents ranged from 0.5 – 1.5 g/kg depending on the induction step; whereas the average daily consumption in adults ranged from 0.8 – 2.2 g/kg depending on the animal. These intake levels routinely produce blood alcohol levels ~ 80 mg/dL, the legal limit in the United States for humans operating a motor vehicle (Sawyer et al. 2014). Average alcohol consumption levels and blood alcohol levels for individual animals are shown in Table 1. Control animals were age-matched, housed in the same facility and exposed to identical conditions as the alcohol self-administering animals, but did not have access to alcohol.

Fecal sample collection

Fresh fecal samples were collected from cage trays from all animals under conditions of standard feeding and exposure, or lack thereof, to alcohol. All samples were collected in the morning between roughly 9AM and 10AM. Samples from each animal were collected serially immediately following one another and all groups were collected contemporaneously. Alcohol availability was then withheld from the self-administering animals for a period of 5 days and fecal samples collected in the same manner. There were no observable signs of withdrawal during this period. Samples were immediately flash frozen and stored at –80°C for future analysis.

DNA isolation and sequencing

DNA was extracted from 200 mg of the fecal samples from each subject using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) following manufacturer's protocols. Concentration of the DNA samples was measured using a NanoDrop™ 1000

spectrophotometer (Thermo Scientific, Waltham, MA). Amplification and sequencing of the V4 region was performed as previously described (Yasuda et al. 2015; Yatsunenko et al. 2012). Briefly, 25 µg of genomic DNA from each sample was amplified using HotMaster Taq DNA Polymerase and HotMasterMix (Quantabio, Beverly, MA) and primers designed to cover the V4 region of 16S rRNA incorporating Illumina adapters and a barcode sequence (515F: GTGCCAGCMGCCGCGGTAA; 806R: GGACTACHVGGGTWTCTAAT). Standard cycling conditions were used: initial denaturation at 94°C for 3 minutes; 30 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 60 seconds, and elongation at 72°C for 5 minutes; and a final elongation step at 72°C for 10 minutes.

Amplicons were quantified (Caliper LabChipGX; PerkinElmer, Waltham, MA), pooled in equimolar concentrations, and size selected (375–425 bp) (Pippin Prep; Sage Sciences, Beverly, MA) to reduce non-specific amplification products from host DNA. Final quality control checks and quantification were performed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA) using a 175-bp paired-end protocol according to the manufacturer's specifications with addition of 5% PhiX.

16S rRNA sequence processing

Following standard quality control and disaggregation, reads were processed using a data curation pipeline in QIIME (Caporaso et al. 2010). First, paired-end reads were joined and size selected to reduce non-specific amplification. These reads were then grouped into operational taxonomic units (OTUs) based on sequence similarity. OTUs were subsequently assigned to an established taxonomy using the Greengenes database release 13_5 (McDonald et al. 2012). Approximately 30,000 reads per sample were obtained. OTUs with fewer than 8 reads and 20% prevalence were excluded from downstream analysis. Data was rarefied to the minimum library size using total sum scaling (TSS). MaAsLin (Morgan et al. 2012) was used for multivariate analysis to identify associations between microbiome abundance and factors of interest including alcohol exposure and various confounding factors (e.g. age and weight). LEfSe (Segata et al. 2011) was used for univariate analyses targeting alcohol exposure only. A maximum false discovery rate (corrected p-value significance threshold) determined using the Benjamini-Hochberg correction was set at 10%. Data visualization and initial statistical analysis was conducted using MicrobiomeAnalyst (Dhariwal et al. 2017).

Alpha richness and diversity from fecal samples collected from control rhesus macaques and animals with chronic access to alcohol before and after abstinence was calculated using the Chao1 (Chao and Mark 1993) and Shannon (Shannon 1948) measures. Differences between the groups were calculated using the non-parametric Mann-Whitney test for the unpaired control and alcohol (baseline or abstinent) groups and the Wilcoxon test for the paired alcohol groups before and after a period of abstinence.

Metabolomic analysis

Fecal samples collected as above were lyophilized and weight equivalents were used for untargeted metabolomic analyses by Metabolon, Inc (Morrisville, NC) using a UPLC-

MS/MS and GC-MS platform. A full accounting of the commercialized process for isolating, identifying, and characterizing metabolites by Metabolon can be found elsewhere (Evans et al. 2009; Shin et al. 2014; Zierer et al. 2018). Briefly, samples are extracted using aqueous and organic solvents with added recovery controls. Small molecules are isolated first by methanol precipitation under vigorous shaking followed by centrifugation. Extract was then separated into an acidic positive-ion condition optimized for hydrophilic compounds, an acidic positive-ion condition optimized for hydrophobic compounds, a basic negative-ion condition with a C18 column, and a negative ionization following elution from an HILIC column. Following Metabolon procedure, fixed injection controls were added.

UPLC-MS/MS was performed using ACQUITY UPLC (Waters, Milford, MA) and a Finnigan LTQ FT mass spectrometer (Thermo Scientific, Waltham, MA). GC-MS was performed using a Finnigan TRACE DSQ GC/MS (Thermo Scientific, Waltham, MA). Compounds were identified by comparison to libraries of purified standards based on retention time/index (RI), mass to charge ratio (m/z), and chromatographic data (Dehaven et al. 2010; Evans et al. 2009). Pairwise comparisons between alcohol drinking animals before and after abstinence or alcohol drinking and control animals were calculated with paired t-tests or Welch's two-sample t-tests respectively with Benjamini-Hochberg correction.

Results

The gut microbiome is stable in rhesus macaques across demographics after four years of age

Initial analyses explored whether there were significant differences in the gut microbiome resulting from demographic factors (e.g. age, body weight, history). Using LEfSe (Segata et al. 2011), no significant differences were found between the younger and older animals at any taxonomic level either when compared alone or in combination with alcohol drinking status (Table S1). MaAsLin (Morgan et al. 2012) was also used to investigate age*alcohol interactions, but again there were no significant effects detected. Age at time of study for alcohol drinkers was also inexorably linked to duration of alcohol exposure. Because no age difference was observed for alcohol drinkers before or after a period of abstinence, there was likewise no difference associated with duration of alcohol exposure. It remains formally possible that age and drinking duration have opposite effects that resulted in canceling each other out, but there was no evidence to suggest this to be the case.

Various other demographic factors were also considered including weight, birth location of animals, and housing room. No significant effect from any of these was expected and none was identified. Additional fecal microbiome data collected from a contemporary cohort of rhesus macaques (Yasuda et al. 2015) was also incorporated at this time and compared to the control animals in the present study. Although these animals had more variable backgrounds and were demographically more diverse, they had been housed at the same location for a minimum of two years and fed the same diet, but were older (ages 12–22) females. The raw reads generated from the previous study were incorporated into the same bioinformatics pipeline and processed concomitantly with the present samples. Again, no significant differences in microbiome composition were observed between these animals and the control animals of the present study. For the remaining analyses in the paper, all microbiome

studies were conducted conflating the age and other demographic distinctions into the three primary groups: control animals, without any exposure to alcohol; alcohol drinking animals, animals with alcohol self-administration histories that were actively consuming alcohol at the time of fecal collection; and alcohol abstinent animals, the same animals with alcohol self-administration histories with samples collected following a five day abstinence period where alcohol was not available.

The gut microbiome is substantially altered in rhesus macaques following chronic alcohol self-administration

The gut microbiome of individual animals did not vary systematically and meaningfully within specific groups (Fig 1A). Overall, animals showed similar patterns with the greatest abundance of Bacteroidetes and Firmicutes. Tenericutes, Actinobacteria, Proteobacteria, Spirochaetes, and other phyla were irregularly distributed among animals and at much lower levels. Taxa richness and evenness, measured by alpha-diversity, was significantly different among the three groups (Fig 1B and 1C). The greatest diversity was observed in control animals, while alcohol drinking animals showed the most restricted microbial communities. Following abstinence diversity was somewhat recovered in animals with a history of alcohol drinking, but still not to the levels seen by control animals.

Beyond the general composition of the microbiome, specific taxa also were observed to be differentially expressed between the groups. Univariate analyses implemented using LEfSe (Segata et al. 2011) identified taxa significantly differentially expressed among the three group (Table 2). Following correction for multiple comparisons, 8 phyla (Fig 2) and 13 families (Fig 3) were identified as showing significant group effects. Broadly speaking, three patterns were observed: alcohol-ever, similar relative abundance in alcohol drinking and abstinent animals but different from controls; alcohol-present, similar relative abundance in controls and alcohol abstinent animals but different from active alcohol drinking animals; and recovering, intermediate relative abundances in alcohol abstinent animals between significantly different controls and alcohol drinking animals

“Alcohol-ever” taxa were characterized by consistent differences between control and alcohol-drinking animals regardless of abstinence periods. The patterns suggest that taxa abundance is reduced following exposure to alcohol and does not rebound. This could be due to the fact that taxa are effectively eradicated or simply that growth is slow compared to the time scales that were considered. There were perhaps surprisingly few of these taxa (i.e. phylum: Euryarchaeota, Tenericutes, Lentisphaerae, Verrucomicrobia; family: R4_45B, Victivallaceae, RFP12, Christensenellaceae, Methanobacteriaceae, RF16). Many more taxa were entirely recovered with abstinence, “alcohol-current” taxa dependent only on alcohol drinking status at time of sample collection (i.e. phylum: Proteobacteria, Firmicutes, Bacteroidetes; family: Helicobacteraceae, Streptococcaceae), or were at intermediate levels during the abstinence period, “recovering” taxa (i.e. phylum: Spirochaetes; family: Spirochaetaceae). Most of these taxa were less abundant in animals drinking alcohol compared to the controls or even the same animals following a period of abstinence. The primary exception to this was the phylum Firmicutes, particularly the family

Streptococcaceae, which was both of high absolute and relative abundance in alcohol drinking animals.

The findings from individual taxa reflect the previous understandings generated from the diversity analyses. In alcohol drinking animals, abundance in most taxa is reduced and Firmicutes dominates the microbiome, while in control animals there are both more taxa represented and they are more abundant. The ratio of Firmicutes to Bacteroidetes, one common measure in gut microbiome studies, is elevated in alcohol drinking animals compared to both control animals and following a period of abstinence. It is perhaps relevant that despite the absence of statistically significant major effects there did appear to be more change occurring in the younger cohort of animals than in the older cohort (Fig S1). It is tempting to relate this back to length of alcohol exposure, although the study remains underpowered and lacks the necessary design to be confident in this interpretation.

Within both of the major phyla, Firmicutes and Bacteroidetes, there is also evidence of a remodeling of communities at lower levels. In Firmicutes the dominant taxa, Streptococcaceae, is significantly elevated in alcohol drinking animals. This decreases following a period of abstinence and Lachnospiraceae becomes significantly elevated. This greater abundance of Lachnospiraceae is not observed in control animals, however, although there a significantly higher level of Christensenellaceae is observed than in animals with any history of alcohol drinking. Within Bacteroidetes, the patterns of taxa recovery following an abstinence period are also observed at the family level. RF16 remains significantly lower in animals with an alcohol history even after a period of abstinence, while Bacteroidaceae returns to control levels. Interestingly, Paraprevotellaceae, Porphyromonadaceae, and Prevotellaceae all show elevated levels following a short period of abstinence that are not seen when comparing control animals with the alcohol drinking animals under their normal, alcohol available, circumstances.

Across the various taxa and analyses two commonalities stand out. First, the microbiome of animals drinking alcohol is reduced in complexity and dominated by Firmicutes. Almost every other taxa is more abundant in control animals, resulting in a significantly greater diversity overall. Second, the microbiome of alcohol drinking animals following a period of abstinence tends to represent a middle ground between when they are actively drinking alcohol and control animals without any prior alcohol exposure.

The fecal metabolome of rhesus macaques is significantly altered by alcohol drinking and abstinence

The relative levels of 410 biochemical compounds were identified in this study (Table S2). For each compound, control animals and animals exposed to alcohol were compared using a Welch's two-sample t-test. After correction for multiple testing, there were no statistically significant different metabolites between the groups. However, when the same metabolites were compared using paired t-tests between animals while drinking compared to a period of abstinence, 22 metabolites were significantly changed (Table 3, Figure 4).

More than one third of the metabolites significantly changed following abstinence were fatty acids, a significant overrepresentation compared to all detectable metabolites (8 out of 22

compared to 60 out of 410; $p = 0.0079$, Fishers Exact test). Glucose-6-phosphate and fructose-6-phosphate, part of energy utilization upstream of the Krebs cycle, show patterns of decreased levels in animals while drinking alcohol compared to following a period of abstinence. Unlike the pattern observed among the fatty acids, there is also a significantly higher relative concentration detected in control animals compared to alcohol drinking. Again, the glycolysis pathway is overrepresented among the overrepresented among significantly different metabolites (2 out of 22 compared to 7 out of 410; $p = 0.049$, Fishers Exact test), though this does not survive correction for multiple tests.

These results are impacted greatly by intra-group variation. A two way repeated measures ANOVA showed that only a paucity of the metabolites varied significantly by age or age * alcohol interaction (Table S3). More generally, however, the alcohol drinking animals following abstinence show much higher levels of variation than either of the other two groups or when compared between groups. While these patterns hold for both the younger cohort of animals and the older cohort of animals, they are more pronounced in the younger animals (Fig 4). This suggests that the effects of the abstinence period, whether the lack of alcohol presence in particular or the change in relative dietary caloric intake more generally, has a stronger effect on younger animals. Apart from the alcohol intake, there is also an observed increase in free amino acids in older animals compared to the younger animals. This likely reflects an age related difference in protein degradation that is independent of alcohol exposure.

Discussion

This study further validates the rhesus macaque as an animal model of the human gut microbiome. The predominance of the phyla Firmicutes and Bacteroidetes and the relative sparsity of Actinobacteria and Verrucomicrobia found in the current study in fecal samples is consistent with the luminal content of previously studies of the macaque gut microbiome (Yasuda et al. 2015). Similarly, the more specific patterns of taxa presence and abundance at the family level were consistent with those observed in both previous studies of macaques (Handley et al. 2012; McKenna et al. 2008; Yasuda et al. 2015) and humans on more traditional (i.e. non-Western) diets (Yatsunenko et al. 2012). This parallel also extends to the effects of alcohol consumption with the reductions in microbiome diversity and increase in relative abundance of Firmicutes in rhesus macaques similar to previous studies of humans with alcohol use disorders (Leclercq et al. 2014; Mutlu et al. 2012).

The use of rhesus macaques allows for controlled interventions that can be difficult in humans because of confounding factors. Here we tested a relatively short five day abstinence period and observed a largely intermediate phenotype, measures of alpha-diversity were intermediate between those seen for controls and during active drinking and the Firmicutes to Bacteroidetes ratio normalized. Relatively few taxa remained at similar, significantly lower, levels than controls in alcohol drinking animals before and after the abstinence period. It remains unproven if these differences would more completely normalize given a longer period of abstinence, but we would hypothesize that they would. This suggests that the drinking procedure employed here reflects a more moderate consumption pattern.

Differences in the fecal metabolome were largely associated with shifts from drinking to abstinence, although it seems likely that this study was statistically underpowered to detect differences from control animals. The dominant changes in metabolites that were observed were associated with fatty acids and metabolites from the glycolysis pathway. The effects of microbiome on behavior are believed to be mediated, at least in part, from changes in small molecules resulting from microbial metabolism (Cryan and Dinan 2012; Sampson and Mazmanian 2015), though the specific changes observed have not previously been demonstrated to have any effects. It is possible, rather, that they are reflective of a shift in dietary homeostasis. While the overall diets of both alcohol consuming and control animals were roughly isocaloric and animals in both groups maintained similar weights without consistent between group deviations including during periods of drinking and abstinence, the relative contribution of calories from differing sources was variable.

These studies offer both a validation of human studies of the effects of alcohol consumption on the microbiome and a well-controlled model for further investigations. In human studies, a separation was observed during withdrawal with some individuals showing greater microbiome dysbiosis that coupled with increased psychological symptoms associated with relapse (Leclercq et al. 2014). The reason for these disparate groups was not immediately obvious; the differences were not correlated with amount of alcohol consumed or history of alcohol consumption. A rhesus macaque model offers the opportunity to investigate factors that may be driving these differences. Secondary insults to the microbiome may have additional additive effects that make a more severe form of alcohol dependence more likely. In particular, a Western diet may exaggerate or predispose the gut microbiome towards dysbiosis (Ley et al. 2006). This model may also present a more translationally relevant opportunity to test interventional approaches to alcohol use disorders such as probiotics or fecal transplantation. More generally, it allows for the dissociation the direct effects of alcohol from co-occurring behavioral and physiological confounds.

Conclusion

Here we demonstrate that chronic consumption of alcohol has specific effects on the microbiome of rhesus macaques characterized by a decrease in microbial diversity with enrichment of relative abundance of Firmicutes. These can be attributed directly to the alcohol consumption and are not the result of concomitant changes to diet or other environmental factors, but reflect a microbiota and gastrointestinal activity common to primates and translationally relevant to humans. Surprisingly, these effects were partially if not wholly ameliorated following a relatively short 5-day period of abstinence, suggesting that the specific effects observed here are the direct effects of alcohol. While there changes may be specific to the conditions of this model of chronic but moderate-level alcohol consumption, it offers an important perspective on the variables at play in the investigation of the role the gut microbiome plays in alcohol use and its potential as a therapeutic target.

Supplementary Figure 1. Comparison of relative abundances of Firmicutes (left) and Bacteroidetes (right) in individual animals while self-administering alcohol and after a 5-day period of abstinence. Older and younger cohorts are identified by color.

Supplementary Figure 2. Comparison of scaled abundance of metabolites with a significant main effect of alcohol exposure in individual animals while self-administering alcohol and after a 5-day period of abstinence. Older and younger cohorts are identified by color.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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All animals studies were performed in accordance with the guidelines of the National Academies Guide for Care and Use of Laboratory Animals, 8th Edition (National Research Council 2011). All protocols were approved by the Harvard Medical School Institutional Animal Care and Use Committee prior to commencement of animal studies.

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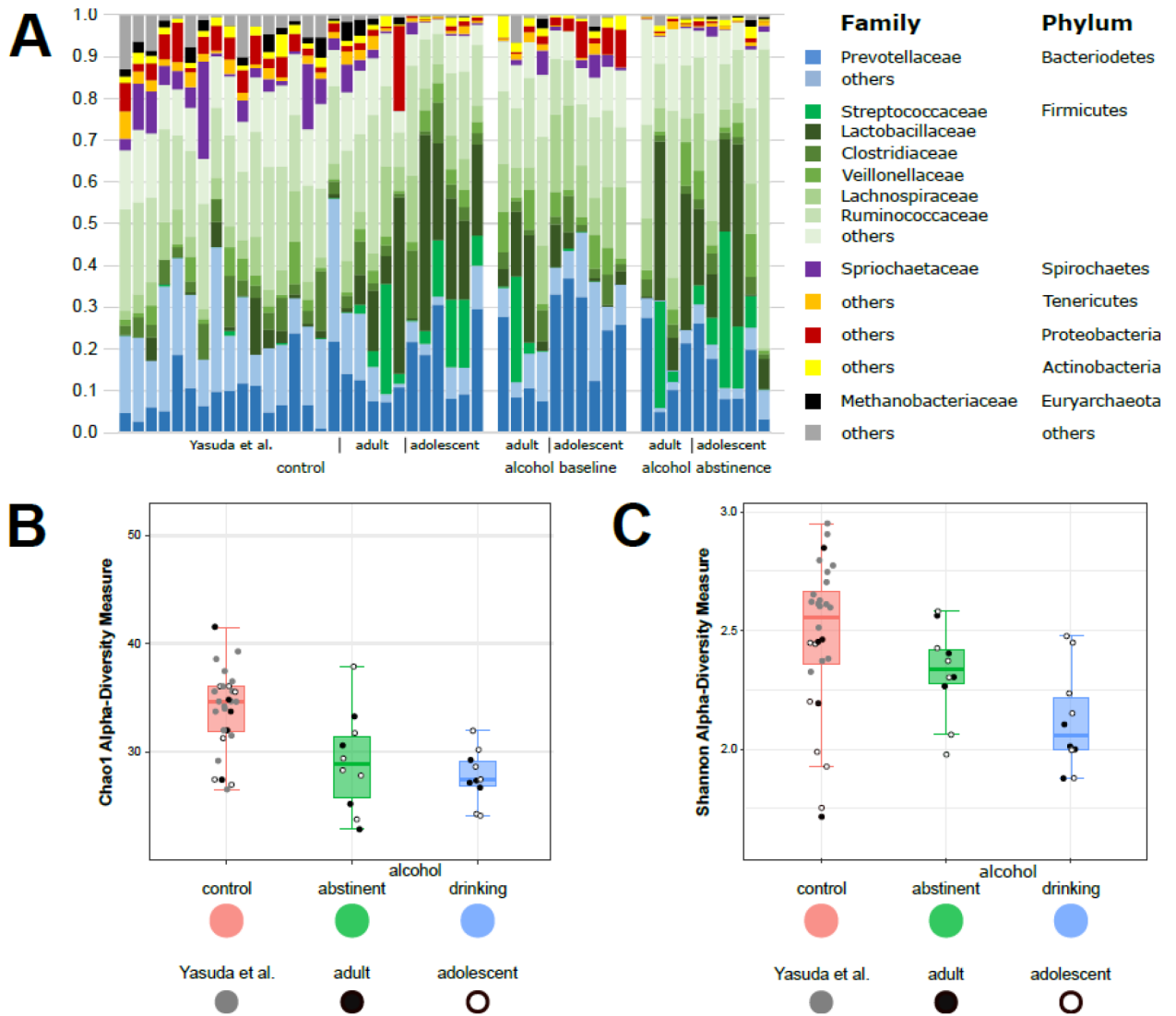


Figure 1.

Influence of alcohol on the gut microbiome in rhesus macaques. (A) Family-level relative abundance of intestinal microbiota in the stool of rhesus macaques without exposure to alcohol (left), with long-term free access to alcohol (right), and following a period of abstinence from alcohol (middle). (B) Alpha diversity from fecal samples collected from control rhesus macaques and animals with chronic access to alcohol before and after abstinence using the Chao1 measure (control vs. alcohol abstinent: two-tailed Mann-Whitney $p = 0.0076$; control vs. alcohol drinking: two-tailed Mann-Whitney $p = 0.0003$; alcohol drinking vs. alcohol abstinent: two-tailed Wilcoxon 0.2983). (C) Alpha diversity using the Shannon measure (control vs. alcohol abstinent: two-tailed Mann-Whitney $p = 0.0414$; control vs. alcohol drinking: two-tailed Mann-Whitney $p = 0.0047$; alcohol drinking vs. alcohol abstinent: two-tailed Wilcoxon 0.0969).

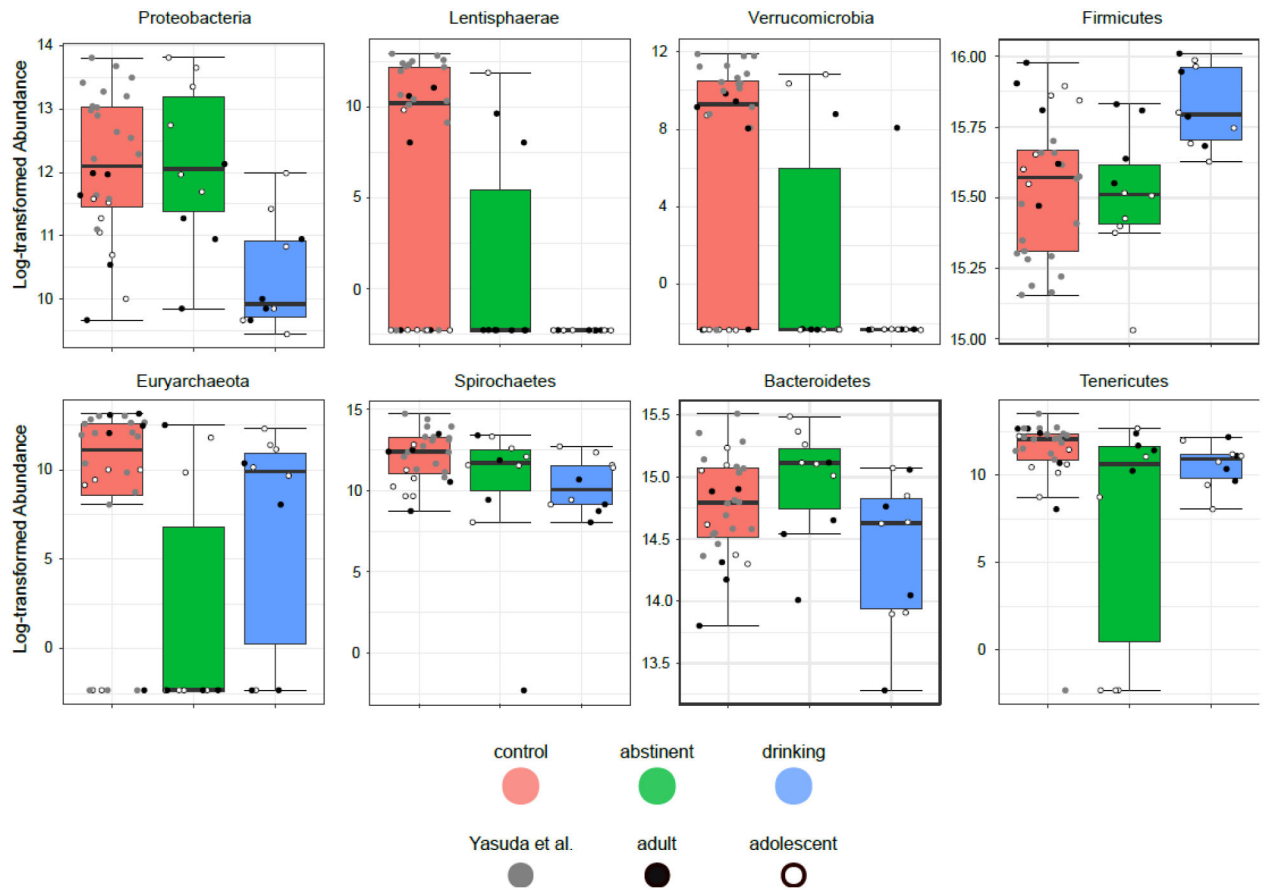


Figure 2.

Influence of alcohol on gut taxa in rhesus macaques at the Phylum level. For each phylum, a box-and-whisker plot is shown of the log-transformed abundance observed in control (left), alcohol-abstinent (center), and alcohol-drinking (right) rhesus macaques. Animals from the Yasuda et al. study are shown in gray, animals from the older cohort in solid black, and animals from the younger cohort in white with black outline. Significance was determined using LEfSe (Segata et al. 2011) and is shown in Table 2.

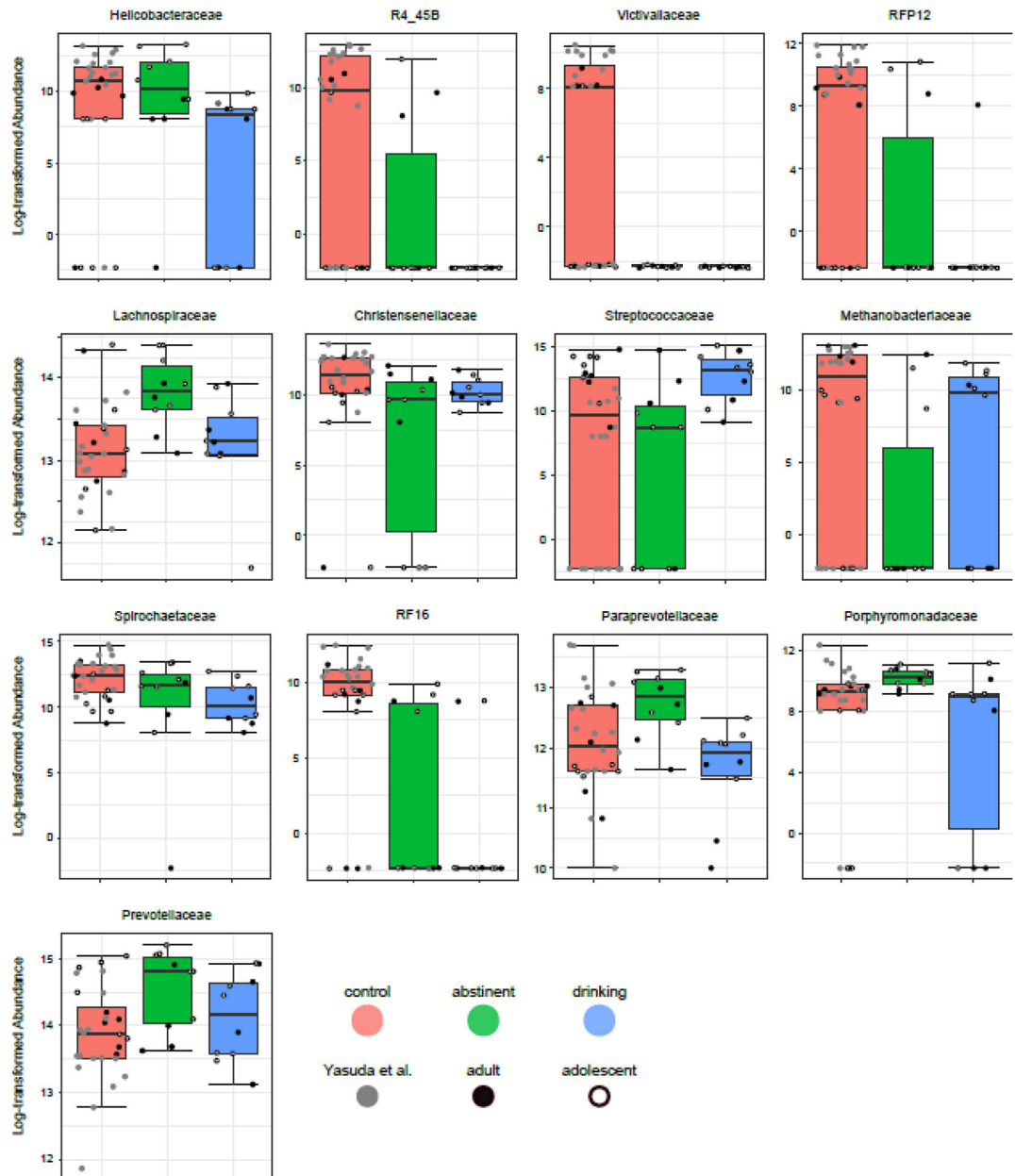


Figure 3.

Influence of alcohol on gut taxa in rhesus macaques at the Family level. For each family, a box-and-whisker plot is shown of the log-transformed abundance observed in control (left), alcohol-abstinent (center), and alcohol-drinking (right) rhesus macaques. Animals from the Yasuda et al. study are shown in gray, animals from the older cohort in solid black, and animals from the younger cohort in white with black outline. Significance was determined using LefSe (Segata et al. 2011) and is shown in Table 2.

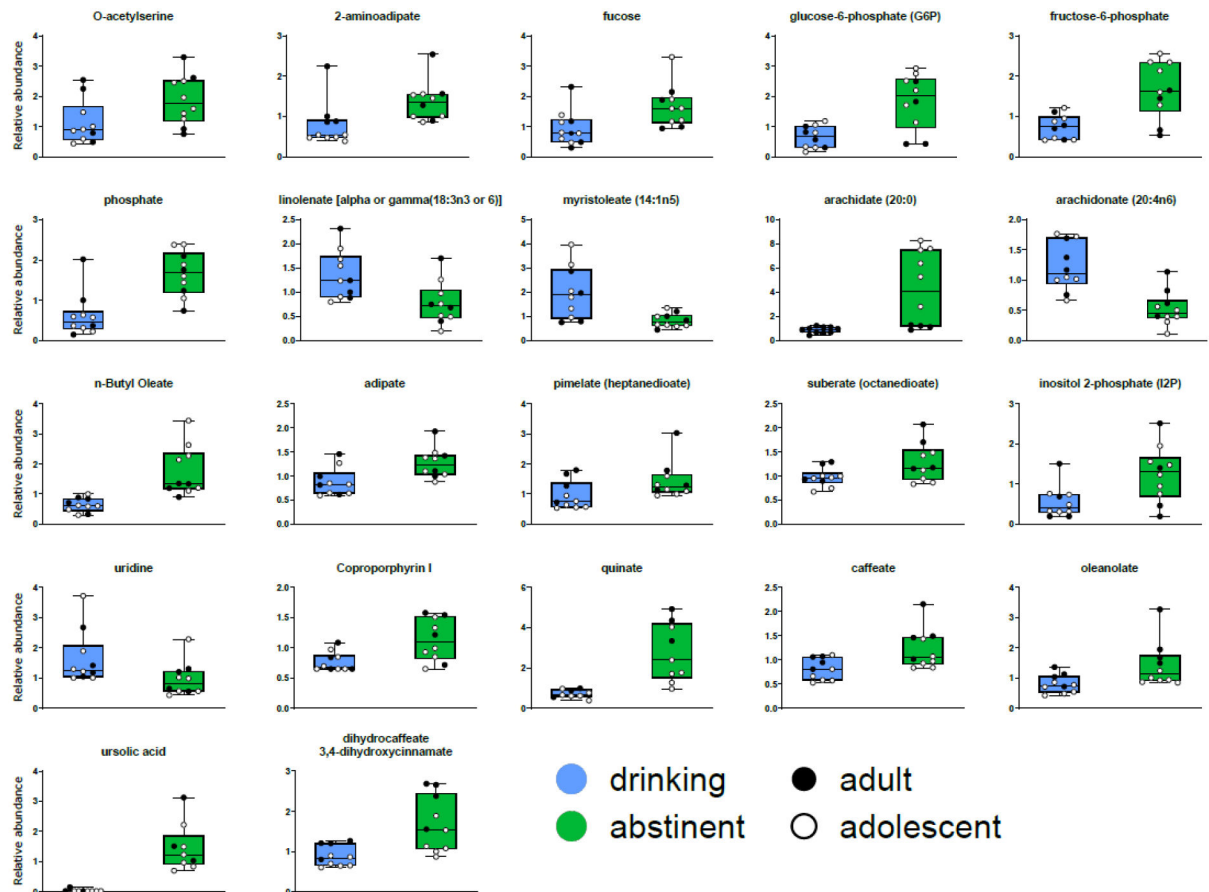


Figure 4.

Influence of alcohol on fecal metabolites in rhesus macaques. A box-and-whisker plot of scaled abundance is shown in alcohol-drinking (left), and alcohol-abstinent (right) rhesus macaques. Animals from the older cohort in solid black and animals from the younger cohort in white with black outline. Significant main-effect of alcohol exposure was calculated a paired t-test with Benjamini-Hochberg correction for multiple test comparisons and is shown in Table 3.

Table 1.

Individual alcohol consumption and blood alcohol levels (BAL) for rhesus macaques used in this study. Values for adult alcohol group reflect intakes/BALs from the year prior to sample collection. Values for the adolescent alcohol group reflect intakes from the first 3 months of alcohol availability during step-wise induction; BAL was determined on the final day of 1.5 g/kg intake.

Monkey	Group ^a	Average Alcohol g/kg (\pm SEM)	Average BAL mg/dl (\pm SEM)
MM-33	Adult alcohol	2.2 (0.08)	128.5 (1.3)
MM-162	Adult alcohol	1.8 (0.01)	105.5 (3.7)
MM-247	Adult alcohol	1.6 (0.07)	88.6 (2.3)
MM-267	Adult alcohol	1.8 (0.09)	106.4 (5.0)
MM-107	Adolescent alcohol	1.0 (0.05)	104.4 (-)
MM-118	Adolescent alcohol	0.9 (0.06)	109.2 (-)
MM-119	Adolescent alcohol	0.9 (0.05)	111.4 (-)
MM-121	Adolescent alcohol	1.0 (0.05)	105.4 (-)
MM-128	Adolescent alcohol	0.9 (0.05)	101.4 (-)
MM-132	Adolescent alcohol	1.0 (0.06)	107.3 (-)

Table 2.

Phyla and families significantly (FDR < 0.10) different in control, alcohol-drinking, and alcohol-abstinent rhesus macaques.

Phylum	Family	P-value	FDR	Alcohol			LDAscc
				Control	Abstinent	Drinking	
Proteobacteria		0.0021	0.0082	299194	344045	48834	5.17
	Helicobacteraceae	0.0047	0.0292	96206	143037	4726	4.84
Lentisphaerae		0.0008	0.0049	95193	7876	630	4.67
	R4_45B	0.0009	0.0105	87092	7876	630	4.64
Verrucomicrobia		0.0006	0.0049	31056	9767	0	4.19
	Victivallaceae	0.0043	0.0292	8102	0	0	3.61
	RFP12	0.0006	0.0097	31056	9767	0	4.19
Firmicutes		0.0029	0.0087	5737015	5575929	7508822	5.99
	Lachnospiraceae	0.0052	0.0292	596588	1108066	603970	5.41
	Christensenellaceae	0.0216	0.0648	178009	43478	32136	4.86
	Streptococcaceae	0.0229	0.0648	351067	299307	1008192	5.55
Eury archaeota		0.0361	0.0467	155505	45684	48204	4.74
	Methanobacteriaceae	0.0454	0.0909	145828	42848	39067	4.73
Spirochaetes		0.0272	0.0408	448285	203214	82861	5.26
	Spirochaetaceae	0.0272	0.0712	448285	203214	82861	5.26
Bacteroidetes		0.0389	0.0467	2808421	3454316	2092628	5.83
	RF16	0.0002	0.0060	52435	3151	1260	4.41
	Paraprevotellaceae	0.0111	0.0419	246647	386894	138626	5.09
	Porphyromonadaceae	0.0073	0.0354	22842	35602	12917	4.05
	Prevotellaceae	0.0417	0.0909	1332478	2405797	1632325	5.73
Tenericutes		0.0169	0.0337	177446	73094	64272	4.75

Table 3.

Fecal biochemicals significantly (FDR < 0.05) different between alcohol drinking animals before and after 5 day abstinence

Biochemical	Platform	PubChem	CAS	Mean Values		P-value	FDR
				Alcohol			
				Abstinent	Drinking		
ursolic acid	GC/MS	64945	77-52-1	3.385	0.038	0.0000	0.0006
Isobar: dihydrocaffeate, 3,4-dihydroxycinnamate	GC/MS			1.679	0.885	0.0000	0.0034
uridine	LC/MS Neg	6029	58-96-8	0.952	1.641	0.0001	0.0078
inositol 2-phosphate (I2P)	GC/MS			1.249	0.554	0.0001	0.0101
phosphate	GC/MS	1061	7664-38-2	1.657	0.623	0.0002	0.0135
caffeate	GC/MS	689,043	331-39-5	1.219	0.812	0.0002	0.0135
n-Butyl Oleate	GC/MS	5354342	142-77-8	1.757	0.639	0.0004	0.0180
quinat	GC/MS		77-95-2	5.382	0.733	0.0004	0.0180
adipate	GC/MS	196	124-04-9	1.269	0.872	0.0004	0.0180
suberate (octanedioate)	LC/MS Pos	10457	505-48-6	1.277	0.972	0.0008	0.0324
oleanolate	GC/MS	10494	508-02-1	1.416	0.797	0.0013	0.0405
fucose	GC/MS	3034656	2438-80-4	1.675	0.944	0.0013	0.0405
fructose-6-phosphate	GC/MS		103213-47-4	1.658	0.736	0.0014	0.0405
2-aminoadipate	GC/MS	469	542-32-5	1.370	0.789	0.0015	0.0405
O-acetylserine	GC/MS		66638-22-0	1.879	1.134	0.0015	0.0405
myristoleate (14:1n5)	LC/MS Neg	5281119	544-64-9	0.834	1.960	0.0017	0.0430
pimelate (heptanedioate)	GC/MS	385	111-16-0	1.436	0.950	0.0021	0.0490
linolenate [alpha or gamma; (18:3n3 or 6)]	LC/MS Neg			0.773	1.352	0.0022	0.0490
Coproporphyrin I	LC/MS Neg		69477-27-6	1.130	0.768	0.0023	0.0490
arachidate (20:0)	GC/MS	10467	506-30-9	4.238	0.871	0.0025	0.0497
glucose-6-phosphate (G6P)	GC/MS		103192-55-8	1.841	0.651	0.0027	0.0497
arachidonate (20:4n6)	LC/MS Neg	444899	506-32-1	0.526	1.222	0.0027	0.0497