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Long-lasting microbial dysbiosis and altered enteric neurotransmitters in adult rats following adolescent binge ethanol exposure

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Abstract

Human alcoholism and ethanol exposure of adult mice cause acute microbial dysbiosis. Adolescent binge drinking is common, but the effect of adolescent ethanol exposure on the adult microbiome and enteric neurotransmitters has not been studied. In the current study, male Wistar rats received adolescent intermittent ethanol (AIE) treatment, and fecal samples were collected on postnatal day (P)54 and P95 for bacterial 16S rRNA amplicon sequencing. Cecal tissue was collected on P95 for analysis of innate immune and neurotransmitter marker expression. At the genus level, AIE treatment altered the relative abundance of several microbes, including decreased relative abundance of *Dehalobacterium* and *CF231* (a member of the Paraprevotellaceae family) that persisted into adulthood. Across aging, the relative abundance of several microbes was altered in both control- and AIE-treated rats. At P95, AIE exposure was associated with increased cecal serotonin levels and reduced choline acetyltransferase gene expression. Taxonomic shifts at P54 and at P95 suggest that AIE causes both immediate and lasting microbial dysbiosis. The lasting microbial dysbiosis was accompanied by alterations of enteric neurotransmitters.

Keywords

adolescence; aging; alcohol; microbiome; serotonin

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AUTHOR CONTRIBUTIONS

RPV and FTC were responsible for the study concept and design. All authors contributed equally to the data preparation and analysis. All authors were involved in drafting and editing the manuscript and have approved the final version for publication. We thank Dr M Andrea Azcarate-Peril for assistance in microbiome data analysis. We thank Jennie Vaughn for assistance with editing of the manuscript.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

1 | INTRODUCTION

Emerging studies find gut microbes impact brain function and communicate with the brain, impacting health¹ as well as developmental disorders.² Multiple studies report that alcohol exposure alters intestinal microbiome causing an imbalance in gut microbiota.³ Dysbiosis is found in alcohol-dependent human subjects,^{4,5} humans with alcohol-induced liver disease,^{5,6} and adult rodent models of ethanol exposure.^{7–10} Rodent studies employing a variety of ethanol exposure paradigms reveal that changes to the intestinal microbiome contribute to gut permeability, endotoxemia, and innate immune induction, which may in turn drive disease pathology,^{8,9,11} including craving and other psychological symptoms of alcohol dependence.⁴ These findings suggest that alcohol pathology may in part be related to alterations in the microbiome.

Adolescence is a developmental period of growth and pubertal maturation that marks the transition from childhood to adulthood and, in humans, often coincides with alcohol binge drinking.¹² Studies using rat models find adolescent ethanol exposure causes lasting consequences that persist into adulthood.¹³ Many studies find adolescent alcohol exposure causes lasting changes in adults that are not found with the same alcohol exposure in adulthood.¹³ Some of the behavioral effects of adolescent alcohol exposure overlap with those in emerging microbiome studies. Nonetheless, although alcohol exposure is known to alter adult microbiome, little is known regarding the adolescent microbiome and potential lasting effects of adolescent ethanol exposure on the adult enteric microbiome.

We investigated the impact of alcohol exposure during adolescence on the intestinal microbiome. Previous animal studies investigating the effect of alcohol on the intestinal microbiome have primarily focused on the immediate effects of ethanol exposure (ie, 1 day after ethanol exposure) in adult subjects. However, adult behaviors are altered by adolescent alcohol exposure,¹³ and many of these overlap with emerging studies of microbiome impacting brain function.¹ Adolescent intermittent ethanol (AIE) exposure, which models adolescent binge drinking, causes persistent changes in brain neurotransmitters, including cholinergic¹⁴ and serotonergic neurons,¹⁵ two neurotransmitters common in the enteric nervous system. Therefore, the purpose of this study was to elucidate the immediate and lasting effect of AIE on the rat microbiome and enteric neurotransmitter systems. Using 16S rRNA amplicon sequencing, we assessed two ages, late adolescence (ie, postnatal day [P]54) immediately following the conclusion of AIE treatment and adulthood (ie, P95) after 41 days of abstinence, to look for persistent dysbiosis. The findings presented here reveal that AIE exposure causes some transient but other persistent microbial dysbiosis as well as long-term alterations in enteric neurotransmitters.

2 | MATERIALS AND METHODS

2.1 | Animals and AIE treatment paradigm

Male Wistar rats bred and reared at the University of North Carolina at Chapel Hill were used in this study. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animals were treated in an Assessment and Accreditation of Laboratory Animal

Care accredited facility. The protocol was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill (Protocol Number: 17–253).

On P21, male Wistar rats were randomly assigned to either (a) AIE or (b) water control (CON) conditions ($n = 8$ – 10 subjects per group). To minimize the impact of litter variables, no more than one subject from a given litter was assigned to a single experimental condition. From P25 to P54, AIE subjects received a single daily intragastric (ig) administration of ethanol (5.0 g/kg, 20% ethanol, w/v) in the AM on a 2-day on/2-day off schedule, and CON subjects received comparable volumes of water on an identical schedule (see Figure 1A). Tail blood was collected from AIE- and CON-treated subjects 1 hour after treatment to assess blood ethanol concentrations (BECs) on P38 (AIE: 162 mg/dL [± 13]) and P54 (AIE: 3.0 mg/dL [± 0.2]) using a GM7 Analyzer (Analox, London, UK). Control subjects did not evidence detectable BECs consistent with water treatment. Body weights were measured through AIE to the conclusion of experimentation. All subjects evidenced dramatic body weight increases across age (main effect of age: $P < .01$). We did not observe an effect of treatment ($P > .9$) on body weight (Figure S1).

2.1.1 | 16S rRNA amplicon sequencing—Fresh fecal samples (~ 200 mg per time point/subject) were collected 1 hour following the conclusion of AIE treatment (P54) and at termination of the experiment on P95 (Figure 1A). Samples were collected using sterile swabs and sterile vials, immediately frozen in liquid nitrogen, and stored at -80°C . Isolation of total DNA from fecal samples was carried out as previously described.^{16,17} Briefly, 16S rRNA amplicon sequencing was performed by the University of North Carolina at Chapel Hill Microbiome Core Facility (NIH Funding # P30 DK34987). Total bacterial DNA was amplified using primers (forward, $5'$ -GTGCCAGCMGCCGCGGTAA- $3'$; reverse, $5'$ -GGACTACHVGGGTWTCTAAT- $3'$) targeting the V3-V4 region of the 16S rRNA gene and overhang adapter sequences appended to the primer pair for compatibility with Illumina index and sequencing adapters.¹⁸ The master mix used was $2\times$ KAPA HiFi HotStart Ready-mix (KAPA Biosystems, Wilmington, MA), and each 16S rRNA amplicon was purified using AMPure XP reagent (Beckman Coulter, Indianapolis, IN). Each sample was then amplified using a limited cycle PCR program, adding Illumina sequencing adapters and optional dual-index barcodes (index 1[i7] and index 2[i5]; Illumina, San Diego, CA) to the amplicon target. The final libraries were again purified using AMPure XP reagent, quantified, and normalized prior to pooling. The DNA library pool was denatured with NaOH, diluted with hybridization buffer, and heat denatured before loading on the MiSeq reagent cartridge and on the MiSeq instrument (Illumina). Automated cluster generation and paired-end sequencing with dual reads were performed.

Analyses of 16S rRNA amplicon-sequencing data were carried out using the QIIME pipeline (QIIME v.1.8.0)¹⁹ using the Greengenes database as previously described.^{16–18} The amplicon size was 291 bp. Briefly, the combined raw sequencing data plus metadata were demultiplexed and filtered for quality control. Sequences were grouped into Operational Taxonomic Units (OTUs) using UCLUST.²⁰ After taxonomic assignment of OTUs (mean sequencing depth [reads/sample] = $170\,807.25$), sequences were aligned and phylogenetic trees built.²¹ OTU tables were used to perform alpha and beta diversity calculations,

measurements that were used with sample metadata to create graphic visualizations for scientific interpretation. A combination of Unifrac significance and principle coordinate analysis (PCoA) using Fast Unifrac²² was used to compare samples based on relevant parameters.

2.1.2 | Perfusion and tissue collection—At the conclusion of experimentation on P95, subjects were anesthetized with a euthanasia dose of sodium pentobarbital (100 mg/kg, ip) and transcardially perfused with 0.1M phosphate-buffered saline (PBS, pH 7.4). Cecal tissue was collected, flushed with sterile PBS, rapidly frozen in liquid nitrogen, and stored at −80°C for RNA and protein isolation.

2.1.3 | RNA/DNA extraction and real-time quantitative PCR—Total RNA was extracted from each cecal sample by pulverization using a Bessman Tissue pulverizer (ThermoFisher Scientific, Austin, TX) followed by homogenization in TRI reagent (Sigma-Aldrich, St. Louis, MO) using FastPrep® Lysing Matrix tubes (MP Biomedicals, Solon, OH) following the single-step method of RNA isolation.²³ RNA quality and concentration were determined using the NanoDrop 1000 (ThermoFisher Scientific). Cecal mRNA (1.0 µg) was reverse transcribed into cDNA using a commercially available kit (Life Technologies, Carlsbad, CA). DNA was isolated from the cecum using the Powerbead RNA isolation kit (Qiagen, Hilden, Germany) following manufacturer's instructions for isolation of both RNA and DNA. Fecal DNA was used at a final concentration of 5.0 ng/µL in the quantitative PCR (qPCR) reactions. qPCR reactions were run on a BioRad CFX system (BioRad, Hercules, California). SYBER Green PCR Master Mix (Life Technologies) was used for qPCR. The qPCR was run with an initial activation for 10 minutes at 95°C, followed by 40 cycles of denaturation (95°C, 15 s), annealing/extension (57°C–58°C, 1 min), and melt curve. The primer sequences are presented in Table 1. Differences in primer expression between groups are expressed as cycle time (Ct) values normalized with a housekeeping gene, and relative differences between groups are calculated and expressed as the percent difference relative to CONs.

2.1.4 | Protein isolation and enzyme-linked immunosorbent assay—Cecal tissue samples (100 mg) were pulverized using a Bessman Tissue pulverizer (ThermoFisher Scientific) followed by homogenization in cold lysis buffer (100-mg tissue/mL; 20-mM Tris, 0.25M sucrose, 2.0-mM EDTA, 10-mM EGTA, 1.0% Triton X-100) containing Complete Mini protease inhibitor cocktail (one tablet/10 mL; Roche Diagnostics, Indianapolis, IN) using FastPrep® Lysing Matrix tubes (MP Biomedicals). Homogenates were clarified by centrifugation at $21\,000 \times g$ for 40 minutes at 4°C. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to determine levels of serotonin (Eagle Biosciences, Nashua, NH) and glutamate (Abnova, Taiwan) in the cecal tissue homogenate according to the manufacturer's protocol.

2.1.5 | Statistical analysis—Statistical analysis was performed using SPSS (Chicago, IL). Student *t* tests were used to assess BECs, qPCR, and ELISA data. To assess the effect of AIE on relative abundance of bacterial taxa at the different ages (ie, P54 and P95), Shapiro-Wilk tests were first performed to determine if the data were normally distributed to

determine appropriateness of Student *t* tests. A *P* value of .05 was set as the significance level for rejection of the null hypothesis during Shapiro-Wilk testing. Paired *t* tests and Student *t* tests were used if the data were normally distributed, whereas Wilcoxon signed-rank tests were used for assessing age-associated microbiome changes, and Mann-Whitney-Wilcoxon *U* tests were used to assess microbiome changes between CON- and AIE-treated animals when the data were determined not to be normally distributed. Since performing multiple comparisons can increase the incidence of type I errors, the Benjamini-Hochberg procedure (B-H Critical) for controlling false positives was calculated (Thissen et al.,²⁴). An individual statistical test was considered significant if the *P* value was less than the B-H Critical value (false discovery rate threshold = 0.1). Analysis of body weight and select bacteria across ages and treatment conditions were assessed using repeated measure analyses of variance. All values are reported as mean $\pm$ SEM.

3 | RESULTS

3.1 | Adolescence and adult intestinal microbiome diversity

Studies in humans report significant differences in the microbiome between healthy children (ie, 1–4 years of age) and adults (ie, 21–60 years of age) consistent with microbiota changes during development.²⁵ We studied late adolescent Wistar rats (P54) and compared them with adults (P95) using 16S rRNA amplicon sequencing. From adolescence to adulthood, 21 genera were altered in the CON subjects and 18 genera altered in the AIE subjects. Seven of the genera changed with age across both CON- and AIE-treated subjects, including *Blautia*, *Bacteroides*, *Parabacteroides*, *Coprobacillus*, Enterobacteriaceae Unclassified, Rikenellaceae Unclassified, and *Proteus*, suggesting that some maturational changes were conserved across the two treatment conditions (Tables 3 and 4). Of the 14 genera that were uniquely altered across aging in the CONs, eight increased with age, including Bacillales Other Other (513%, *P* = .084), *Allobaculum* (342%, *P* = .049), Victivallaceae Unclassified (383%, *P* = .018), and RF32 Unclassified Unclassified (207%, *P* = .002). In contrast, the relative abundance of six genera were uniquely reduced across aging in CON subjects, including *CF231* (–87%, *P* = .027), *Porphyromonas* (–100%, *P* = .059), Streptophyta Unclassified Unclassified (–99%, *P* = .036), and *Christensenella* (–50%, *P* = .027), indicative of age-related maturational changes in the microbiome (Table 3). Alpha diversity, which provides an index of bacterial diversity within each sample, was assessed across four parameters, including observed species (Figure 1B) and Chao1 (Figure 1C) to assess OTU richness, and phylogenetic diversity (PD) whole tree (Figure 1D) to assess phylogenetic differences as well as Shannon (Figure 1E) as a measure of OTU diversity as a function of richness and abundance. We did not observe an effect of AIE or aging on measures of alpha diversity across samples. Thus, rat maturation from adolescence to adulthood includes alterations in microbiota genera but little or no change in alpha diversity. We next assessed beta diversity using UniFrac as a measure of bacterial community relatedness. Weighted UniFrac distances using control groups at P54 and P95 as the reference groups revealed that neither AIE nor aging affected measures of beta diversity (Figure 2A,B).

3.2 | Effect of AIE exposure and age on the relative abundance of intestinal microbes at the phylum level

For taxonomic analysis of the rat intestinal microbiome, we focused on changes at the phylum and genus levels. The most abundant phyla were Firmicutes and Bacteroidetes, comprising an average of 63% and 29% of the total intestinal bacteria in late adolescence, and 59% and 33% in adulthood, respectively (Figure 3A). While AIE treatment did not significantly alter the relative abundance of Firmicutes (Figure 3B) or Bacteroidetes (Figure 3C) at either time point, we did observe a subtle age-related trend for reduced Firmicutes and a trend toward increased Bacteroidetes levels. Populations of Actinobacteria, which was the third most abundant phylum, showed a significant change across age in the AIE group, being 307% more abundant in late adolescent AIE-treated subjects ($P = .08$) before returning to CON levels at P95 (Figure 3D). Thus, there were only subtle changes in abundance of Firmicutes and Bacteroidetes by age or AIE, while AIE treatment led to a modest increase of Actinobacteria phylum immediately following ethanol exposure that resolved in abstinent maturation to adulthood.

3.3 | AIE exposure causes long-term alterations of the intestinal microbiome

While AIE treatment did not affect the intestinal microbiome at the phylum level, we observed AIE-induced changes in the relative abundance of multiple intestinal bacteria at the genus level (Table 2 and Figure S2). We found that AIE exposure altered the relative abundance of 21 bacterial genera in late adolescence and eight bacterial genera in adulthood (Table 2). Interestingly, two of the genera assessed, *Dehalobacterium* (Figure 4) and *CF231* (Table 2), were altered at P54 that persisted into adulthood. The 45% reduction of *Dehalobacterium* relative abundance at P54 and 36% reduction at P95 suggests a persistent effect of AIE exposure on this bacterial genus (Figure 4A). AIE exposure decreased relative abundance of Lachnospiraceae Unclassified at P54 by 43% ($P = .035$; Figure 4B) and [*Ruminococcus*] by 48% ($P = .006$; Figure 4C), while *Allobaculum* was increased by 19-fold ($P = .016$; Figure 4D) before returning to CON levels at P95. In contrast, relative abundance of *Christensenella* and *Streptococcus* was not affected by AIE exposure at P54 but was increased at P95 by 225% ($P = .019$; Figure 4E) and 256% ($P = .100$; Figure 4F), respectively, relative to CONs. The phylum of Actinobacteria was increased by AIE at P54 returning to CON levels at P95. Interestingly within the Actinobacteria phylum, *Bifidobacterium* and *Rothia* abundance had opposite changes following AIE (Figure 4). Abundance of *Bifidobacterium* and *Rothia* did not change across age in the CON subjects. In contrast, AIE treatment increased the relative abundance of *Bifidobacterium* by 430% at P54 (Figure 4G) returning to CON levels by P95, whereas *Rothia* was unchanged at P54 but increased to 248% of CONs at P95 (Figure 4H) in the AIE-treated subjects, relative to CONs.

Within the Bacteroidetes phylum, which constituted 30% to 40% of the microbiome at both ages, AIE caused a 57% decrease of *Prevotella* ($P = .020$; Figure 5A) and a 68% decrease of *Paraprevotella* ($P = .043$; Figure 5B) at P54 that returned to CON levels by P95. Interestingly, AIE treatment caused a 95% decrease in the relative abundance of *CF231* ($P = .002$) at P54 that persisted to P95 ($P = .023$; Figure 5C). In contrast, AIE-treated animals evidenced relative abundance increases at P54 of Bacteroidales Unclassified Unclassified

(235%, $P = .067$; Figure 5D), *Butyricimonas* (223%, $P = .041$; Figure 5E), and [*Prevotella*] (324%, $P = .011$; Figure 5F); the latter of which is a unique form of unclassified *Prevotella* that returned to CON levels by P95. Recently, Tsuruya and colleagues²⁶ reported that human alcoholics have decreased levels of a group of ethanol-metabolizing intestinal bacteria (ie, *Ruminococcus*, *Collinsella*, *Prevotella*, *Coriobacterium*, and *Bifidobacterium*) that may contribute to the accumulation of gut acetaldehyde, which is a toxic metabolite of alcohol. Therefore, we determined the summed relative abundance of this group of “acetaldehyde accumulators” in our microbiome analysis. We found no effect of AIE treatment on the summed relative abundance of the acetaldehyde accumulators at either P54 (CON, 12%; AIE, 10%) or P95 (CON, 15%; AIE, 15%). In sum, AIE treatment altered the relative abundance of 21 intestinal bacteria at P54 and eight bacterial genera at P95. Several bacteria modified by AIE just after treatment returned to CON levels during abstinent maturation into adulthood; however, AIE treatment reduced the relative abundance of *CF231* (Bacteroidetes) and *Dehalobacterium* (Firmicutes) in late adolescence that persisted into adulthood, suggesting lasting AIE-induced alterations of the intestinal microbiome.

While most microbiome studies have focused on the effects of ethanol on bacterial communities, Yang and colleagues²⁷ reported that alcohol exposure is also associated with alterations of the intestinal fungal community (ie, the mycobiota) in mice and alcohol-dependent humans. Thus, we next performed qPCR for *Candida albicans*, a species of yeast previously shown to be increased in mouse fecal samples collected 24 hours following the conclusion of 8 weeks of the Lieber-DeCarli model of chronic ethanol exposure.²⁷ We extend these studies with the observation that at P95, 41 days following AIE termination at P54, there was a 4.1-fold increase of *Candida albicans* in fecal samples of adult AIE-treated animals, relative to age-matched CONs ($P = .014$; Figure 6). Thus, similar to the persistent AIE-induced alterations in microbial communities, AIE treatment led to long-term increases of intestinal fungi (ie, *Candida albicans*) in adulthood.

3.4 | AIE and developmental age-related changes of the intestinal microbiome

Of the 12 genera that were uniquely altered across aging in the AIE-treated subjects, nine increased with age, including *Dorea* (1136%, $P = .109$), *Corynebacterium* (178%, $P = .055$), *Streptococcus* (119%, $P = .078$), Lachnospiraceae Unclassified (49%, $P = .014$), *Holdemania* (907%, $P = .059$), *Sutterella* (356%, $P = .039$), *Bilophila* (565%, $P = .016$), and *Aggregatibacter* (717%, $P = .059$). In contrast, we observed a reduction in the relative abundance of *Bifidobacterium* (−76%, $P = .016$), Prevotellaceae Unclassified (−95%, $P = .035$), and [*Paraprevotellaceae*] [*Prevotella*] (−63%, $P = .008$; Table 4). In some cases, the AIE age-related changes were driven by effects of AIE on the microbiome at P54 that returned to baseline CON levels by P95 (eg, *Bifidobacterium*, *Prevotella*, and Lachnospiraceae Unclassified) or alternatively by delayed effects of AIE exposure (eg, *Streptococcus*; Figures 4 and 5). Together, these data reveal ethanol exposure-induced changes by AIE that return to CON levels without alcohol treatment, consistent with ethanol diet-driven microbiome changes that over time return to basal CON levels.

3.5 | AIE treatment causes long-term alterations of neurotransmitter marker expression in the adult cecum

Alcoholic liver disease and alcoholism have been suggested to alter gut permeability and induce inflammation.^{28,29} Therefore, we next assessed innate immune signaling and tight junction protein genes in cecal tissue collected from CON- and AIE-treated animals on P95. In AIE-treated animals, there was no significant change in mRNA expression of the proinflammatory signaling genes *Tnfa*, *Hmgbl*, *Tlr4*, or *Nox2* in the adult cecum. Additionally, mRNA expression of the tight junction proteins *Occludin* and *Claudin-1* was unchanged in adults following AIE treatment (Figure S3). Thus, proinflammatory genes and genes regulating tight junctions are not persistently modified in P95 adult rats following AIE exposure, consistent with recovery from any AIE-induced changes.

The gut is highly innervated and previous studies have found gut-microbiota interact with the enteric neurotransmitter serotonin³⁰ and motility of the gut.²⁹ Therefore, we assessed mRNA expression of genes involved in neurotransmitter synthesis and transport. Expression of the cecal cholinergic neuron marker choline acetyltransferase (*Chat*) mRNA was decreased by 51% in the cecal tissue of adult AIE-treated animals at P95, relative to CONs ($P = .019$). The majority of serotonin formed in the body is in the gut, with both enterochromaffin cells and enteric nervous system serotonergic neurons forming gut serotonin.³⁰ Analysis of serotonin levels revealed that AIE caused an 18% increase in cecal serotonin levels ($P = .001$) in adult AIE-treated animals, relative to CONs. However, tryptophan hydroxylase 1 (*Tph1*) mRNA expression was unaffected at P95 by AIE treatment (Figure 7). Assessment of serotonin transporter (*Sert*) mRNA, which is expressed by most epithelial cells,³¹ revealed a 48% reduction in the cecal tissue of adult AIE-treated animals at P95, relative to CONs ($P = .023$). Glutamate, an amino acid neurotransmitter, was reduced 13% by AIE ($P = .022$; Figure 7B). Thus, adolescent ethanol exposure may contribute to persistent changes to enteric nervous system signaling via altered neurotransmitter levels.

4 | DISCUSSION

To our knowledge, this is the first study to assess the immediate and long-term effects of adolescent binge ethanol exposure on the intestinal microbiome and markers of enteric neurotransmitters. Assessment of the microbiome 1 hour following the conclusion of AIE revealed decreased relative abundance of several microbes from the phylum Firmicutes (eg, *Dehalobacterium* and Lachnospiraceae Unclassified), Bacteroidetes (eg, *CF231*, *Paraprevotella*, and *Prevotella*), and Actinobacteria (eg, *Corynebacterium*) as well as increased relative abundance of other microbes, including *Allobaculum* (Firmicutes), *Bifidobacterium* (Actinobacteria), and *Butyricimonas* (Bacteroidetes). There is accumulating evidence that levels of *Bifidobacterium* increase in response to various pathologies, including alcoholic hepatitis³² and inflammatory bowel disease³³ that may represent a compensatory response to equilibrate the microbiome milieu. Many of these returned to CON levels by P95. However, the AIE-induced decrease in the relative abundance of *Dehalobacterium* and *CF231* persisted into adulthood, consistent with adolescent ethanol exposure causing long-lasting microbial dysbiosis. We also found that AIE treatment caused microbial taxonomic shifts that did not manifest until adulthood, including increased relative

abundance of *Christensenella* (Firmicutes), *Streptococcus* (Firmicutes), and *Rothia* (Actinobacteria). Interestingly, AIE also caused a long-term increase of *Candida albicans*, a highly abundant fungal species previously reported to be acutely increased by alcohol in mice and humans.²⁷ Across maturation from P54 to P95, we observed shifts in the relative abundance of several microbes independent of AIE consistent with age-associated changes of the microbiome from late adolescence to adulthood. In addition to AIE alterations in microbiome and increased fungus, we found that AIE altered cecal neurotransmitters. Acetylcholine contributes to gut motility, and AIE reduced adult cecal *Chat* mRNA could alter function. Increases in serotonin levels and reduced serotonin transporter (*Sert*) mRNA, but not *Tph1*, are consistent with increased enterochromaffin cell serotonin synthesis and reduced epithelial cell uptake and metabolism, which could also alter gut function. Together, these data reveal that adolescent binge ethanol exposure causes long-lasting microbial and fungal dysbiosis as well as alterations of intestinal neurotransmitter systems in adulthood.

Numerous studies have investigated the acute effects of alcohol on the microbiome in humans^{4–6} and rodent models.^{7–10,34} We did not observe an effect of AIE or aging on measures of alpha diversity and beta diversity. Our observation of an acute abundance increase of the phylum *Actinobacteria* at P54 is similar to findings reported in adult mice immediately following the conclusion of exposure to the Lieber-DeCarli ethanol diet.⁸ Our findings of reduced relative abundance of Lachnospiraceae Unclassified and increased *Allobaculum* at P54 are consistent with findings in adult mice exposed to ethanol liquid diet.³⁴ Similarly, Lowe and colleagues⁸ reported decreased family-level abundance of Lachnospiraceae and Moraxellaceae acutely in adult female mice exposed to the Lieber-DeCarli diet. Reductions of Lachnospiraceae are also observed in human subjects with alcohol-related liver cirrhosis.⁶ Several members of the Lachnospiraceae family are described as producers of short-chain fatty acids, including butyrate,^{35–37} that are thought to contribute to intestinal homeostasis and exert anti-inflammatory effects.^{38,39} We report that AIE reduced the relative abundance of several members of the Lachnospiraceae family immediately following the conclusion of AIE, which may contribute to the observed microbial dysbiosis. Indeed, feeding of butyrate-producing Lachnospiraceae to NLRP12 KO mice exposed to a high-fat diet increased intestinal microbial diversity and dampened inflammatory signaling.³⁹ Similar to our findings in late adolescence, prior rodent studies reported that ethanol exposure did not acutely affect relative abundance of *Streptococcus*, whereas we observe increased *Streptococcus* abundance in adulthood. Human studies report increased levels of *Streptococcus* in alcohol-dependent patients without liver disease⁴⁰ and in patients with alcohol-induced liver disease²⁶ and have further shown that levels of *Streptococcus* positively correlate with severity of alcoholic hepatitis.⁴¹ In addition to lasting microbial dysbiosis, AIE was associated with long-term mycobiota alterations as levels of fecal *Candida albicans* mRNA were increased in adulthood. Similar to our data, levels of this highly abundant fungal species have been reported to be increased by alcohol in mice and humans.²⁷ Together, these data suggest that adolescent binge ethanol treatment causes microbial and fungal dysbiosis that continues into adulthood and may contribute to an altered adult intestinal milieu.

Age is recognized as an important factor contributing to the composition of the intestinal microbiome in both rodents⁴² and humans.⁴³ While some studies report fluctuations in the

composition of the human microbiome early in development that stabilizes in childhood,⁴⁴ others report that the intestinal microbiota undergoes alterations throughout adulthood.⁴⁵ Consistent with the latter study, we found increased relative abundance of Enterobacteriaceae,⁴⁵ *Bacteroides*,^{45,46} and *Allobaculum*⁴⁶ as well as decreased *Prevotella*⁴⁷ and *Blautia*⁴⁶ in the CON group is consistent with the reported age-related changes of these genera in humans. The loss of maturational changes in *Butyricimonas* and *CF231* in the AIE group suggests that adolescent binge ethanol exposure may contribute to premature aging of the intestinal microbiome. In contrast, *Christensenella* was significantly decreased by age in CONs but not in AIE-treated animals, suggesting that AIE may also paradoxically prevent normal age-related changes in the rat microbiome. Thus, binge drinking during adolescence appears to alter maturation of the intestinal microbiome across aging.

The intestinal microbiome is recognized as an important contributor to enteric health and neurotransmitter biosynthesis.⁴⁸ Previous studies report acute intestinal immune induction and diminished tight junction protein expression in ethanol-treated mice and humans with alcohol use disorder.^{11,28,49} In the present study, we observed that AIE treatment did not cause lasting alterations of innate immune (ie, *Tnfa*, *Hmgb1*, *Tlr4*, or *Nox2*) or tight junction protein (ie, *Occludin* and *Claudin1*) genes. This is consistent with data from our laboratory showing that immune activation with lipopolysaccharide persists in brain but not periphery (ie, liver and serum).⁵⁰ Interestingly, assessment of the enteric neurotransmitter systems revealed a long-term reduction of *Chat* mRNA, which is an acetylcholine synthesizing enzyme and highly abundant in the cecum, in the adult cecum following AIE.⁵¹ This observation parallels the AIE-induced reduction of ChAT protein and mRNA in the basal forebrain of the CNS.¹⁴ Glutamatergic neurons are also present in the gut, and glutamate has been implicated in the regulation of gut motility and secretion.⁵² AIE exposure decreased glutamate levels in the cecum of adult AIE-treated subjects, which might contribute to ethanol-induced reductions of intestinal motility.⁵³ Further, we observed increased serotonin levels in the cecum of adult AIE-treated animals that were accompanied by reduced gene expression of the serotonin transporter *Sert*. Consistent with our data, adolescent rats exposed to sole-source ethanol beginning in adolescence and continuing for 60 to 180 days increased 5-HT histochemistry in the jejunum of adult rats.⁵⁴ While the behavioral consequences of dysbiosis and altered enteric neurotransmitter systems remain to be fully elucidated, they may contribute to the psychological symptoms (eg, anxiety, depression, and craving) associated with alcohol use disorder.^{4,55} Indeed, microbial dysbiosis, including increased *Butyricimonas* and reduced *Ruminococcus*, is associated with human depression.⁵⁶ In aged mice, microbiota shifts were associated with cognitive deficits and increased anxiety-like behavior.⁵⁷ Further, antidepressant drugs also exert antimicrobial effects that may contribute to the beneficial effects of these pharmacological agents in the treatment of depression.⁵⁸

In summary, our study reveals that AIE treatment, which models human adolescent binge drinking, causes taxonomic shifts in bacterial abundance from late adolescence to adulthood suggesting that AIE causes both immediate and lasting microbial dysbiosis. Adolescent binge ethanol exposure was also associated with fungal dysbiosis as evidenced by increased levels of *Candida albicans* in adulthood. Measures of the enteric neurotransmitter system revealed long-lasting AIE-induced increases of cecal serotonin and reductions of glutamate

and the acetylcholine-synthesizing enzyme *Chat* in adulthood. Across aging, we observed shifts in the relative abundance of several microbes independent of AIE consistent with age-associated changes of the microbiome from late adolescence to adulthood. The persistent enteric dysbiosis and neurotransmitter system alterations may contribute to the physiological and psychological symptoms associated with alcohol use disorders. These data highlight the importance of investigating the long-term consequences of alcohol exposure on the intestinal microbiome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Dinan Cryan JF. The microbiome-gut-brain axis in health and disease. *Gastroenterol Clin North am*. 2017;46(1):77–89. [PubMed: 28164854]
2. Forssberg H Microbiome programming of brain development: implications for neurodevelopmental disorders. *Dev Med Child Neurol*. 2019;744–749. [PubMed: 30868564]
3. Starkel P, Leclercq S, de Timary P, Schnabl B. Intestinal dysbiosis and permeability: the yin and yang in alcohol dependence and alcoholic liver disease. *Clin Sci (Lond)*. 2018;132(2):199–212. [PubMed: 29352076]
4. Leclercq S, Matamoros S, Cani PD, et al. Intestinal permeability, gutbacterial dysbiosis, and behavioral markers of alcohol-dependence severity. *Proc Natl Acad Sci USA*. 2014;111(42):E4485–E4493. [PubMed: 25288760]
5. Mutlu EA, Gillevet PM, Rangwala H, et al. Colonic microbiome is altered in alcoholism. *Am J Physiol Gastrointest Liver Physiol*. 2012; 302(9):G966–G978. [PubMed: 22241860]
6. Chen Y, Yang F, Lu H, et al. Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology*. 2011;54(2): 562–572. [PubMed: 21574172]
7. Forsyth CB, Farhadi A, Jakate SM, Tang Y, Shaikh M, Keshavarzian A. Lactobacillus GG treatment ameliorates alcohol-induced intestinal oxidative stress, gut leakiness, and liver injury in a rat model of alcoholic steatohepatitis. *Alcohol*. 2009;43(2):163–172. [PubMed: 19251117]
8. Lowe PP, Gyongyosi B, Satishchandran A, et al. Alcohol-related changes in the intestinal microbiome influence neutrophil infiltration, inflammation and steatosis in early alcoholic hepatitis in mice. *PLoS One*. 2017;12(3):1–16, e0174544.
9. Mutlu E, Keshavarzian A, Engen P, Forsyth CB, Sikaroodi M, Gillevet P. Intestinal dysbiosis: a possible mechanism of alcohol-induced endotoxemia and alcoholic steatohepatitis in rats. *Alcohol Clin Exp Res*. 2009;33(10):1836–1846. [PubMed: 19645728]
10. Yan AW E Fouts D, Brandl J, et al. Enteric dysbiosis associated with a mouse model of alcoholic liver disease. *Hepatology*. 2011;53(1): 96–105. [PubMed: 21254165]

11. Fouts DE, Torralba M, Nelson KE, Brenner DA, Schnabl B. Bacterial translocation and changes in the intestinal microbiome in mouse models of liver disease. *J Hepatol.* 2012;56(6):1283–1292. [PubMed: 22326468]
12. Windle M, Spear LP, Fuligni AJ, et al. Transitions into underage and problem drinking: developmental processes and mechanisms between 10 and 15 years of age. *Pediatrics.* 2008;121(Suppl 4):S273–S289. [PubMed: 18381494]
13. Crews FT, Vetreno RP, Broadwater MA, Robinson DL. Adolescent alcohol exposure persistently impacts adult neurobiology and behavior. *Pharmacol Rev.* 2016;68(4):1074–1109. [PubMed: 27677720]
14. Vetreno RP, Crews FT. Adolescent binge ethanol-induced loss of basal forebrain cholinergic neurons and neuroimmune activation are prevented by exercise and indomethacin. *PLoS One.* 2018;13(10):1–22, e0204500.
15. Vetreno RP, Patel Y, Patel U, Walter TJ, Crews FT. Adolescent intermittent ethanol reduces serotonin expression in the adult raphe nucleus and upregulates innate immune expression that is prevented by exercise. *Brain Behav Immun.* 2017;60:333–345. [PubMed: 27647531]
16. Allali I, Delgado S, Marron PI, et al. Gut microbiome compositional and functional differences between tumor and non-tumor adjacent tissues from cohorts from the US and Spain. *Gut Microbes.* 2015;6(3): 161–172. [PubMed: 25875428]
17. Devine AA, Gonzalez A, Speck KE, et al. Impact of ileocecal resection and concomitant antibiotics on the microbiome of the murine jejunum and colon. *PLoS One.* 2013;8(8):1–13, e73140.
18. Allali I, Arnold JW, Roach J, et al. A comparison of sequencing platforms and bioinformatics pipelines for compositional analysis of the gut microbiome. *BMC Microbiol.* 2017;17(1):194–210. [PubMed: 28903732]
19. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* 2010; 7(5):335–336. [PubMed: 20383131]
20. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* 2010;26(19):2460–2461. [PubMed: 20709691]
21. Price MN, Dehal PS, Arkin AP. FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS One.* 2010;5(3): 1–10, e9490.
22. Lozupone C, Hamady M, Knight R. UniFrac—an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics.* 2006;7:374–385. [PubMed: 16901353]
23. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. *Nat Protoc.* 2006;1(2):581–585. [PubMed: 17406285]
24. Thissen D, Steinberg L, Kuang D. Quick and Easy Implementation of the Benjamini-Hochberg Procedure for Controlling the False Positive Rate in Multiple Comparisons. *J of Edu and Behav Stat.* 2002;27: 77–83.
25. Ringel-Kulka T, Cheng J, Ringel Y, et al. Intestinal microbiota in healthy U.S. young children and adults—a high throughput microarray analysis. *PLoS One.* 2013;8(5):1–10, e64315.
26. Tsuruya A, Kuwahara A, Saito Y, et al. Ecophysiological consequences of alcoholism on human gut microbiota: implications for ethanol-related pathogenesis of colon cancer. *Sci Rep.* 2016;6:27923–27935. [PubMed: 27295340]
27. Yang AM, Inamine T, Hochrath K, et al. Intestinal fungi contribute to development of alcoholic liver disease. *J Clin Invest.* 2017;127(7): 2829–2841. [PubMed: 28530644]
28. Bishehsari F, Magno E, Swanson G, et al. Alcohol and gut-derived inflammation. *Alcohol Res.* 2017;38(2):163–171. [PubMed: 28988571]
29. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Ann Gastroenterol.* 2015;28(2):203–209. [PubMed: 25830558]
30. Yano JM, Yu K, Donaldson GP, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell.* 2015;161(2): 264–276. [PubMed: 25860609]

31. Wade PR, Chen J, Jaffe B, Kassem IS, Blakely RD, Gershon MD. Localization and function of a 5-HT transporter in crypt epithelia of the gastrointestinal tract. *J Neurosci*. 1996;16(7):2352–2364. [PubMed: 8601815]
32. Wang W, Chen L, Zhou R, et al. Increased proportions of Bifidobacterium and the lactobacillus group and loss of butyrate-producing bacteria in inflammatory bowel disease. *J Clin Microbiol*. 2014;52(2):398–406. [PubMed: 24478468]
33. Taur Y, Xavier JB, Lipuma L, et al. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. *Clin Infect Dis*. 2012;55(7):905–914. [PubMed: 22718773]
34. Bull-Ottersen L, Feng W, Kirpich I, et al. Metagenomic analyses of alcohol induced pathogenic alterations in the intestinal microbiome and the effect of lactobacillus rhamnosus GG treatment. *PLoS One*. 2013;8(1):1–10, e53028.
35. Meehan CJ, Beiko RG. A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. *Genome Biol Evol*. 2014;6(3):703–713. [PubMed: 24625961]
36. Reichardt N, Duncan SH, Young P, et al. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. *ISME j*. 2014;8(6):1323–1335. [PubMed: 24553467]
37. Smith PM, Howitt MR, Panikov N, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science*. 2013;341(6145):569–573. [PubMed: 23828891]
38. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. *Aliment Pharm Ther*. 2008;27(2):104–119.
39. Truax AD, Chen L, Tam JW, et al. The inhibitory innate immune sensor NLRP12 maintains a threshold against obesity by regulating gut microbiota homeostasis. *Cell Host Microbe*. 2018;24(3):364–378. e6 [PubMed: 30212649]
40. Dubinkina VB, Tyakht AV, Odintsova VY, et al. Links of gut microbiota composition with alcohol dependence syndrome and alcoholic liver disease. *Microbiome*. 2017;5(1):141–155. [PubMed: 29041989]
41. Llopis M, Cassard AM, Wrzosek L, et al. Intestinal microbiota contributes to individual susceptibility to alcoholic liver disease. *Gut*. 2016; 65(5):830–839. [PubMed: 26642859]
42. Lees H, Swann J, Poucher SM, et al. Age and microenvironment outweigh genetic influence on the Zucker rat microbiome. *PLoS One*. 2014;9(9):1–11, e100916.
43. Thomas S, Izard J, Walsh E, et al. The host microbiome regulates and maintains human health: a primer and perspective for non-microbiologists. *Cancer Res*. 2017;77(8):1783–1812. [PubMed: 28292977]
44. Borre YE, O’Keeffe GW, Clarke G, Stanton C, Dinan TG, Cryan JF. Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends Mol Med*. 2014;20(9):509–518. [PubMed: 24956966]
45. Odamaki T, Kato K, Sugahara H, et al. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol*. 2016;16:90–102. [PubMed: 27220822]
46. Thevaranjan N, Puchta A, Schulz C, et al. Age-associated microbial dysbiosis promotes intestinal permeability, systemic inflammation, and macrophage dysfunction. *Cell Host Microbe*. 2017;23(4):455–466.
47. Bartosch S, Fite A, Macfarlane GT, McMurdo ME. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl Environ Microbiol*. 2004;70(6):3575–3581. [PubMed: 15184159]
48. Wall R, Cryan JF, Ross RP, Fitzgerald GF, Dinan TG, Stanton C. Bacterial neuroactive compounds produced by psychobiotics. *Adv Exp Med Biol*. 2014;817:221–239. [PubMed: 24997036]
49. Lowe PP, Gyongyosi B, Satishchandran A, et al. Reduced gut microbiome protects from alcohol-induced neuroinflammation and alters intestinal and brain inflammasome expression. *J Neuroinflammation*. 2018;15(1):298–310. [PubMed: 30368255]

50. Qin L, Wu X, Block ML, et al. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia*. 2007;55(5): 453–462. [PubMed: 17203472]
51. Legay C, Faudon M, Ternaux JP. Amines and choline acetyltransferase in rat intestine. *Neurochem Int*. 1983;5(3):275–284. [PubMed: 20487950]
52. Kirchgeßner AL. Glutamate in the enteric nervous system. *Curr Opin Pharmacol*. 2001;1(6):591–596. [PubMed: 11757814]
53. Addolorato G, Montalto M, Capristo E, et al. Influence of alcohol on gastrointestinal motility: lactulose breath hydrogen testing in orocecal transit time in chronic alcoholics, social drinkers and teetotaler subjects. *Hepatology*. 1997;44(16):1076–1081. [PubMed: 9261602]
54. Yakovleva LM, Lubovtseva LA. Dynamics of neurotransmitters in the structures of the rat jejunum during chronic alcohol intoxication. *Bull Exp Biol Med*. 2013;155(1):30–33. [PubMed: 23667865]
55. de Timary P, Starkel P, Delzenne NM, Leclercq S. A role for the peripheral immune system in the development of alcohol use disorders? *Neuropharmacology*. 2017;122:148–160. [PubMed: 28400259]
56. Jiang H, Ling Z, Zhang Y, et al. Altered fecal microbiota composition in patients with major depressive disorder. *Brain Behav Immun*. 2015; 48:186–194. [PubMed: 25882912]
57. Scott KA, Ida M, Peterson VL, et al. Revisiting Metchnikoff: age-related alterations in microbiota-gut-brain axis in the mouse. *Brain Behav Immun*. 2017;65:20–32. [PubMed: 28179108]
58. Macedo D, Chaves Filho AJM, de Sousa CNS, et al. Antidepressants, antimicrobials or both? Gut microbiota dysbiosis in depression and possible implications of the antimicrobial effects of antidepressant drugs for antidepressant effectiveness. *J Affect Disord*. 2017;208: 22–32. [PubMed: 27744123]

FIGURE 1.

Effect of adolescent intermittent ethanol (AIE) exposure on alpha diversity of the rat fecal microbiome. A, graphical representation of the AIE paradigm and experimental design. Male Wistar rats bred and reared at the University of North Carolina at Chapel Hill were used in this study. On the day following birth (postnatal day [P]1), litters were culled to 10 pups with six males and four females retained when possible. Pups remained with their dams in standard clear plastic tubs with shavings until group housing with same-sex littermates at the time of weaning on P21. All subjects were housed in a 20°C temperature and humidity-controlled vivarium on a 12 h/12 h light/dark cycle (light onset at 0700 h) and provided ad libitum access to food and water. B-E, 16S rRNA amplicon sequencing of fecal samples was performed as described in Section 2. Alpha diversity comparisons, which provide an index of within-sample microbial richness and/or diversity, are shown a for rarefaction depth of 12 000 reads per sample. Observed species (B) and Chao1 (C) provide measures of richness, phylogenetic diversity (PD) whole tree (D) provides a measure of phylogenetic richness, and Shannon (E) provides a measure of diversity (richness and abundance/evenness). Boxplot whiskers represent the range of minimum and maximum alpha diversity values within a population

FIGURE 2.

Principle coordinate analysis (PCoA) plots of weighted and unweighted UniFrac measures in control- and adolescent intermittent ethanol (AIE)-treated subjects across age. A, weighted UniFrac PCoA plot showing gut microbiota beta diversity across all groups. B, unweighted UniFrac PCoA plot showing fecal microbiota beta diversity across all groups

FIGURE 3.

Relative abundance of fecal bacterial phyla in control- and adolescent intermittent ethanol (AIE)-treated animals across age. A, the average relative abundance of identified bacterial phyla across CON- and AIE-treated subjects on postnatal day (P)54 and P95 is depicted as the percentage of total bacteria identified per sample. B-D, 16S rRNA amplicon sequencing analysis of the relative abundance of the three most prevalent phyla: B, Firmicutes; C, Bacteroidetes; and D, Actinobacteria. Data are presented as mean % relative abundance $\pm$ SEM. #.05 < P < .1, relative to age-matched CON; ^b effect of age (P < .05) in AIE

FIGURE 4.

Adolescent intermittent ethanol (AIE) exposure alters fecal abundance of genera belonging to the Firmicutes and Actinobacteria phyla. Bacterial 16S rRNA amplicon sequencing analysis of the relative abundance of select bacterial genera within the Firmicutes (A-F) and Actinobacteria (G and H) phyla. Data are presented as mean % relative abundance $\pm$ SEM. Bacterial classifications encapsulated by brackets [] are used to indicate that they are misclassified, meaning incorrect placement in a higher taxonomic rank. #.05 < P < .1, * P < .05, relative to age-matched CON; ^beffect of age (P < .05) in AIE

FIGURE 5.

Adolescent intermittent ethanol (AIE) exposure alters fecal abundance of genera belonging to the Bacteroidetes phylum. Bacterial 16S rRNA amplicon sequencing analysis of the relative abundance of select bacterial genera within the Bacteroidetes (A-F) phylum. Data are presented as mean % relative abundance $\pm$ SEM. Bacterial classifications encapsulated by brackets [] are used to indicate that they are misclassified, meaning incorrect placement in a higher taxonomic rank. * $P < .05$, relative to age-matched CON; ^aeffect of age ($P < .05$) in CON

FIGURE 6.

Adolescent intermittent ethanol (AIE) exposure is associated with increased fecal levels of *Candida albicans* in adulthood. Quantitative PCR assessment of *Candida albicans* in fecal samples collected on postnatal day 95 revealed a significant increase, relative to CONs. qPCR analyses were run in triplicate. Data are presented as mean $\pm$ SEM. * $P < .05$, relative to CONs

FIGURE 7.

Adolescent intermittent ethanol (AIE) treatment alters expression of neurotransmitter markers in the adult cecum. A, quantitative PCR assessment of neurotransmitter genes revealed a significant AIE-induced reduction of *Chat* (51% [$\pm 11\%$]) and *Sert* (48% [$\pm 8\%$]), but not *Tph1* in the adult (postnatal day [P]95) cecum tissue samples, relative to CONs. qPCR analyses were run in triplicate. B, Cecal levels of serotonin and glutamate were determined by commercially available ELISA kits. ELISA analysis revealed a significant 18% ($\pm 4\%$) increase of serotonin and a significant 13% ($\pm 4\%$) reduction of glutamate levels in the cecum of AIE-treated animals in adulthood (P95), relative to CONs. Data are presented as mean $\pm$ SEM. * $P < .05$, relative to CONs

Primers used for real-time qPCR analysis

TABLE 1

Gene	Species	Forward (5'–3')	Reverse (5'–3')
<i>Tnfr</i>	Rat	ATG TGG AAC TGG CAG AGG AG	ACG AGC AGG AAT GAG AAG AAG
<i>Hmgbl</i>	Rat	ATG GCA AAA GGA GAT CCT	ATT CAT CAT CAT CTT CT
<i>Thr4</i>	Rat	GCC GGA AAG TTA TTG TGG	ATG GGT TTT AGG CGC AGA GTT T
<i>Nox2</i>	Rat	TGG AGT GGT GTG TGA ATG CCA GAG	CGG ACA GCG ACT GCT GA
<i>Ocludin</i>	Rat	CAG CAC AAC ACT TAG ACA GTG C	CCT TTC CAC TCG GGC TCA AT
<i>Claudin-1</i>	Rat	CTG GGA GGT GCC CTA CTT TC	CAC CCT TCG CCC ATT TGA GT
<i>Inos</i>	Rat	TGC ACC CAA ACA CCA AGG T	CTC AGC ACA GAG GGC TCA AAG
<i>Il6</i>	Rat	CTG GTC TTC TGG AGT TCC GTT	GGT CTT GGT CCT TAG CCA CTC
<i>Il1β</i>	Rat	GAA ACA GCA ATG GTC GGG AC	AAG ACA CGG GTT CCA TGG TG
<i>Chat</i>	Rat	GCC CAA CCA AGC CAA GCA A	AAA TGT CTT TGC GGG TGC C
<i>Tph1</i>	Rat	CCC ACC TCT GCC TTT CAC ATT	CTC GAC CGG CTT CCT TGA GA
<i>Sert</i>	Rat	CCA CCT TCC CAT ACA TTG T	CTG TCT CCA AGA GTT TCT GC
<i>18s</i>	Rat	CGG GGA ATC AGG GTT CGA TT	TCG GGA GTG GGT AAT TGC G
<i>16s</i>	Bacteria (universal)	TCC TAC GGG AGG CAG CAG T	GAC TAC CAG GGT ATC TAA TCC TGT T
<i>ITS</i>	<i>Candida albicans</i>	TTT ATC AAC TTG TCA CAC CAG A	ATC CCG CCT TAC CAC TAC CG

TABLE 2

Effect of adolescent intermittent ethanol (AIE) exposure across aging (ie, P54 and P95) on the relative abundance of select genera, relative to CONs

Genus	Effect of AIE			
	P54		P95	
	P value	B-H critical	P value	B-H critical
Actinobacteria; Actinomycetales; Corynebacteriaceae; Corynebacterium	↓ 0.054	0.046	0.155	0.042
Actinobacteria; Actinobacteria; Actinomycetales; Micrococaceae; Rothia	0.929	0.100	↑ 0.068	0.019
Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium	↑ 0.054	0.050	0.351	0.065
Bacteroidetes; Bacteroidia; Bacteroidales; [Barnesiellaceae]; unclassified	↑ 0.068	0.058	0.143	0.035
Bacteroidetes; Bacteroidia; Bacteroidales; [Odoribacteriaceae]; Butyrivibrio	↑ 0.041	0.035	0.824	0.092
Bacteroidetes; Bacteroidia; Bacteroidales; [Paraprevotellaceae]; [Prevotella]	↑ 0.011	0.015	0.351	0.069
Bacteroidetes; Bacteroidia; Bacteroidales; [Paraprevotellaceae]; CF231	↓ 0.002	0.004	↓ 0.023	0.008
Bacteroidetes; Bacteroidia; Bacteroidales; [Paraprevotellaceae]; Paraprevotella	↓ 0.043	0.038	0.894	0.096
Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas	↓ 0.072	0.065	0.434	0.073
Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella	↓ 0.020	0.027	0.564	0.081
Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; unclassified	↑ 0.050	0.042	↓ 0.072	0.023
Bacteroidetes; Bacteroidia; Bacteroidales; unclassified; unclassified	↑ 0.067	0.054	0.824	0.088
Firmicutes; bacilli; Lactobacillales; Streptococcaceae; streptococcus	0.790	0.096	↑ 0.100	0.031
Firmicutes; clostridia; Clostridiales; Christensenellaceae; Christensenella	0.424	0.092	↑ 0.019	0.004
Firmicutes; clostridia; Clostridiales; Dehalobacteriaceae; Dehalobacterium	↓ 0.016	0.019	↓ 0.083	0.027
Firmicutes; clostridia; Clostridiales; Dehalobacteriaceae; unclassified	↑ 0.080	0.073	0.143	0.038
Firmicutes; clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus]	↓ 0.006	0.008	0.198	0.054
Firmicutes; clostridia; Clostridiales; Lachnospiraceae; Dorea	↓ 0.083	0.077	0.689	0.085
Firmicutes; clostridia; Clostridiales; Lachnospiraceae; unclassified	↓ 0.035	0.081	0.965	0.100
Firmicutes; clostridia; Clostridiales; Mogibacteriaceae; Anaerovorax	↑ 0.077	0.069	0.285	0.062
Firmicutes; clostridia; Clostridiales; Peptostreptococcaceae; unclassified	↑ 0.068	0.062	0.307	0.077
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Allobaculum	↑ 0.016	0.023	0.267	0.058
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Coprobacillus	↓ 0.007	0.012	0.168	0.046
Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Bilophila	0.286	0.088	↑ 0.056	0.015
Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Aggregatibacter	0.283	0.085	↑ 0.045	0.012
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter	↓ 0.028	0.031	0.180	0.050

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Notes. Fecal samples from CON- and AIE-treated male Wistar rats were collected on the last day of treatment (postnatal day [P]54) and in adulthood on P95. All genera found to be changed by AIE with a $P < .1$ at either age are listed. † indicates increased relative abundance in AIE-treated subjects, relative to CONs; ‡ indicates decreased relative abundance in AIE-treated subjects, relative to CONs. An individual statistical test was considered significant if the P -value was less than the B-H Critical value as indicated in **bold**. Bacterial classifications encapsulated by brackets [] are used to indicate that they are misclassified, meaning incorrect placement in a higher taxonomic rank. *Italic* indicates use of nonparametric Mann-Whitney-Wilcoxon U -tests.

Genera changes across age in CON subjects

TABLE 3

Genus	% change across age	P value	B-H critical
Proteobacteria; Alphaproteobacteria; RF32; unclassified; unclassified	206.71	0.002	0.005
Actinobacteria; Coriobacteriales; Coriobacteriaceae; unclassified	-60.19	0.004	0.010
Firmicutes; clostridia; Clostridiales; Lachnospiraceae; Blautia	345.78	0.006	0.014
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Proteus	4784.6	0.009	0.019
Lentisphaerae; [Lentisphaeria]; Victivallales; Victivallaceae; unclassified	382.7	0.018	0.024
Bacteroidetes; Bacteroidia; Bacteroidales; [Odoribacteraceae]; Butyrivimonas	150.17	0.020	0.033
Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroides	98.12	0.020	0.029
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Coprobacillus	193.18	0.020	0.038
Bacteroidetes; Bacteroidia; Bacteroidales; [Paraprevotellaceae]; CF231	-87.32	0.027	0.043
Firmicutes; clostridia; Clostridiales; Christensenellaceae; Christensenella	-50.29	0.027	0.048
Cyanobacteria; chloroplast; Streptophyta; unclassified; unclassified	-99.44	0.036	0.052
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; unclassified	640.71	0.044	0.057
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Allobaculum	341.72	0.049	0.062
Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas	-99.99	0.059	0.067
Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; unclassified	-37.26	0.062	0.086
Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Parabacteroides	108.57	0.064	0.071
Firmicutes; clostridia; Clostridiales; Dehalobacteriaceae; unclassified	46.84	0.083	0.076
Bacteroidetes; Bacteroidia; Bacteroidales; unclassified; unclassified	77.76	0.084	0.081
Firmicutes; bacilli; Bacillales; other; other	512.72	0.084	0.090
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter	99.93	0.094	0.095
Euryarchaeota; Methanobacteria; Methanobacteriales; Methanobacteriaceae; Methanospaera	-37.07	0.097	0.100

Notes. Fecal samples from male Wistar rat CON subjects were collected in adolescence on postnatal day (P)54 and in adulthood on P95. All bacterial genera that changed across age with a $P < .1$ are listed. Percent change across age was calculated as $(P95-P54)/P54 * 100$. Negative values indicate decreased levels in adults (P95) compared with adolescents (P54); positive values indicate increased levels in adults (P95) compared with adolescents (P54). Data are presented as mean % relative abundance $\pm$ SEM. An individual t test is considered significant if the P value is less than the B-H Critical value. Bacterial classifications encapsulated by brackets [] are used to indicate that they are misclassified, meaning incorrect placement in a higher taxonomic rank.

TABLE 4

Genera changes across age in adolescent intermittent ethanol (AIE)-treated subjects

Genus	% change across age	P value	B-H critical
Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; unclassified	-41.20	0.006	0.026
Bacteroidetes; Bacteroidia; Bacteroidales; [Paraprevotellaceae]; [Prevotella]	-63.25	0.008	0.005
Firmicutes; clostridia; Clostridiales; Lachnospiraceae; Blautia	568.03	0.008	0.011
Firmicutes; clostridia; Clostridiales; Lachnospiraceae; unclassified	48.49	0.014	0.032
Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium	-76.25	0.016	0.016
Proteobacteria; Deltaproteobacteria; Desulfotribionales; Desulfotribionaceae; Bilophila	564.58	0.016	0.021
Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; unclassified	-95.44	0.035	0.037
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Proteus	847.08	0.035	0.042
Proteobacteria; Betaproteobacteria; Burkholderiales; Alcaligenaceae; Sutterella	355.88	0.039	0.047
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; unclassified	472.08	0.052	0.053
Actinobacteria; Actinobacteria; Actinomycetales; Corynebacteriaceae; Corynebacterium	177.68	0.055	0.058
Firmicutes; clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus]	276.90	0.055	0.063
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Holdemania	907.00	0.059	0.068
Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Aggregatibacter	717.14	0.059	0.074
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Coprobacillus	2242.37	0.076	0.079
Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides	180.07	0.078	0.084
Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Parabacteroides	109.04	0.078	0.089
Firmicutes; bacilli; Lactobacillales; Streptococcaceae; streptococcus	118.62	0.078	0.095
Firmicutes; clostridia; Clostridiales; Lachnospiraceae; Dorea	1135.90	0.109	0.100

Notes. Fecal samples from male Wistar rat AIE subjects were collected on the last day of AIE treatment (postnatal day[P]54) and in adulthood on P95. All bacterial genera that changed across age with a $P < .1$ are listed. Percent change across age was calculated as $((P95-P54)/P54) * 100$. Negative values indicate decreased relative abundance in adults (P95) compared with adolescents (P54); positive values indicate increased relative abundance in adults (P95) compared with adolescents (P54). Data are presented as mean % relative abundance $\pm$ SEM. An individual statistical test was considered significant if the P value was less than the B-H Critical value as indicated in **bold**. Bacterial classifications encapsulated by brackets [] are used to indicate that they are misclassified, meaning incorrect placement in a higher taxonomic rank. *Italic* indicates use of nonparametric Wilcoxon sign-rank tests.