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Innate gut microbiota predisposes to high alcohol consumption

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Abstract

Gut microbiota is known to be transferred from the mother to their offspring. This study determines whether the innate microbiota of rats selectively bred for generations as high alcohol drinkers play a role in their alcohol intake. Wistar-derived high-drinker UChB rats (intake 10-g ethanol/kg/day) administered nonabsorbable oral antibiotics *before allowing* access to alcohol, reducing their voluntary ethanol intake by 70%, an inhibition that remained after the antibiotic administration was discontinued. Oral administration of *Lactobacillus rhamnosus* Gorbach–Goldin (GG) induced the synthesis of FGF21, a vagal β-Klotho receptor agonist, and partially re-invoked a mechanism that reduces alcohol intake. The vagus nerve constitutes the main axis transferring gut microbiota information to the brain ("microbiota-gut-brain" axis). Bilateral vagotomy inhibited rat alcohol intake by 75%. Neither antibiotic treatment nor vagotomy affected total fluid intake. A microbiota-mediated marked inflammatory environment was observed in the gut of ethanol-naïve high-drinker rats, as gene expression of proinflammatory cytokines (TNF-α; IL-6; IL-1β) was significantly reduced by nonabsorbable antibiotic administration. Gut cytokines are known to activate the vagus nerve, while vagal activation induces pro-rewarding effects in nucleus accumbens. Both alcoholics and alcohol-preferring rats share a marked preference for sweet tastes—likely an evolutionary trait to seek sweet fermented fruits. Saccharin intake by UChB rats was inhibited by 75%–85% by vagotomy or oral antibiotic administration, despite saccharin-induced polydipsia. Overall, data indicate that the mechanisms that normally curtail heavy drinking are inhibited in alcohol-preferring animals and inform a gut microbiota origin. Whether it applies to other mammals and humans merits further investigation.

KEYWORDS
alcoholism, antibiotics, gut microbiota, microbiota-gut-brain axis

Fernando Ezquer and Maria Elena Quintanilla contributed equally to this study.

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1 | INTRODUCTION

Excessive alcohol consumption represents a major public health problem worldwide.[1] It has been estimated that over 75 million people presenting an alcohol use disorder (AUD) (WHO, 2016).

While AUDs have traditionally been considered to initiate at the brain level, with changes in the brain reward circuit,[2] novel aspects of addictive behavior have also implicated neuroinflammation as an important factor contributing to the perpetuation of chronic alcohol intake and relapse.[3]

1.1 | The microbiota-gut-brain axis

Gut bacteria are new participants in many psychiatric and neurological diseases, including AUDs.[4–7] Studies by Bravo et al. showed that the oral administration of gram-positive *Lactobacillus rhamnosus* JB1 to mice modified GABA α2 mRNA expression in several brain areas,[8] also reducing stress-induced corticosterone, anxiety, and depression-related behavior. Noteworthy, these effects were not observed in vagotomized animals, indicating that the vagus nerve is the main communication system linking the *Lactobacillus* modified gut microbiota with the brain.[8] The microbiota-gut-brain axis has also emerged as a potential new therapeutic target.[7] The vagus nerve contains somatic and visceral afferent fibers entering the brain via the X cranial nerve and extending distally to the ventral tegmental area and the nucleus accumbens.[9] The vagus nerve tract, including its nodose ganglia, expresses GABA and beta-Klotho receptors as well as receptors for the gram-negative bacteria-derived lipopolysaccharide (LPS) and proinflammatory cytokines.[10,11] In rodents, chronic ethanol intake or its administration leads to increased levels of LPS in portal blood,[12,13] along with an increase in serum TNF-α and neuroinflammation, effects that are suppressed by antibiotic administration.[14] Remarkably, the peripheral injection of LPS induces a long-lasting increase in ethanol intake in mice,[15] strongly linking peripheral inflammation to high alcohol intake.

1.2 | Microbiota and alcohol intake

The transplantation for 2 weeks of feces from alcohol-dependent patients to C57BL-6 mice was shown by Zhao et al. to increase the preference of the animals for alcohol solutions over water.[16] Jahvad et al.[17] showed that for outbred Wistar rats born at the authors' facilities, some animals, but not other, showed a marked preference for ethanol, which correlated with a specific composition of gut microbiota of those animals, a finding that suggests that the permanent gut microbiota taxa depend on the genetic endowment of the animals.

Mardones and associates, starting from rats of the original Wistar Institute, selectively bred two substrains; the nondrinker UChA line and the alcohol drinker strain UChB.[18] Following 90 selective breeding generations, the UChB strain was subjected to seven generations of brother–sister mattings, and when given free choice of a water solution or 10% and 20% (v/v) ethanol solutions, they consume up to 10-g ethanol/kg body weight/day.[19]

A large number of studies, in both rodents and humans, have shown that chronic alcohol intake per se alters the composition of their gut microbiota (dysbiosis).[20–24] The transplantation of gut microbiota from alcoholic patients (AUD) to mice generates behavioral changes akin to depression.[16,21,25] In some AUD patients, gut bacteria found at the time of alcohol withdrawal showed changes following 3 weeks of abstinence, thus indicating that alcohol per se can change the gut microbiota composition. However, in other patients, the gut microbiota composition remained mainly constant following abstinence,[6,26,27] suggesting that the gut microbiota taxa either require a longer term alcohol abstinence than 3 weeks or there is an innate alcoholism-predisposing microbiota phenotype.

Several reports have suggested that gut microbiota constitutes a potential co-factor for ethanol-induced peripheral and brain inflammation.[28,29] Studies showed that alcohol and its main oxidative metabolite acetaldehyde alter the intestinal barrier, disrupting tight junctions and promoting intestinal hyperpermeability.[12,13] This allows microbial proinflammatory products, including the gram-negative generated LPS, to translocate from the intestinal lumen into the portal and systemic circulation, which as indicated earlier promotes alcohol intake.[28]

A recent phase I clinical study by Bajaj et al. showed that fecal transplantation from a normal individual (commercially available feces high in *Lachnospiraceae* and *Ruminococcaceae*) to AUD cirrhotic patients led to a marked short-term (15-day) reduction in alcohol craving, alcohol consumption, and serum TNF-α levels, versus placebo.[30]

1.3 | The shared alcohol preference and sweet taste preference

It is well established from animal[31,32] and human[33–35] studies that there is shared preference for alcohol intake as for sweet substances (e.g., sucrose, fructose, and saccharin), referred to as the "sweet tooth of alcoholics." Such an associated preference for both substances may have an evolutionary origin.[36,37] In the wild, animals can detect the odor of alcohol in naturally fermented fruits, allowing them to increase their carbohydrate intake. Evolution has however also developed a firewall against both alcohol inebriation and excess fructose intake, as both can be hepatotoxic.[38–40] Following sugar or alcohol intake, the liver generates fibroblast growth factor-21 (FGF21),[41,42] a hormone that reduces sugar and alcohol intake while increasing dopamine transporter transcription in the nucleus accumbens.[9]

Both alcohol[43,44] and saccharin[45] are known to increase dopamine release in nucleus accumbens, which in the presence of FGF21 will be less rewarding.[9] Because the oral administration of *L. rhamnosus* has been shown to stimulate FGF21 synthesis,[46] changes in gut microbiota may also influence ethanol intake.

Given the above information, studies were conducted to determine the effect of the *innate gut microbiota* and of vagotomy on voluntary alcohol and saccharin intake in alcohol-preferring rats in which gut microbiota alteration or vagus nerve ablation was performed *prior*

to allowing animal access to the preferred solution, thus dissociating the ethanol effect per se (or saccharin per se) from the early intake of the rewarding substance Thus, we hypothesize that (a) in rats selected for their high alcohol intake, both (i) a reduction in gut microbiota diversity by the administration of oral nonabsorbable antibiotics prior to ethanol access and (ii) the prior ablation of the vagus nerve will blunt their high alcohol intake. (b) We further hypothesize that both a reduction in microbiota diversity and vagus nerve ablation will inhibit saccharin intake.

To test the above hypotheses, the goals of the present study were (i) to determine the voluntary intake of an alcohol solution and of a saccharin solution following the prior administration of two oral nonabsorbable antibiotics or of bilateral vagotomy. The study further aims at (ii) determining whether endogenous gut microbiota generates a gut proinflammatory state and (iii) testing whether an increase in hepatic FGF21 gene expression and of systemic FGF21 levels that normally follow the oral administration of *L. rhamnosus* Gorbach–Goldin (LGG) could also reduce voluntary alcohol intake.

1.4 | Experimental

(i) In all experiments, oral antibiotics, vagotomy, and the administration of LGG proceeded prior to allowing ethanol access to the animals; (ii) we sequenced the 16S-subunit of the ribosomal RNA from fecal samples taken from the UChB rats and determined the changes in their microbial diversity and community composition following oral nonabsorbable antibiotic treatment immediately prior to exposure to ethanol; (iii) we determined whether oral antibiotic administration influences inflammatory parameters in the gut of this high alcohol preferring rat; and (iv) we determined whether the administration of LGG influenced rat ethanol intake and altered the hepatic FGF21 gene expression and the systemic levels of FGF21.

Overall, the studies conducted show that modifications of gut microbiota and vagotomy markedly influence the intake of both ethanol and saccharin in a rat model oh high alcohol preference.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult male Wistar-derived rats, selectively bred for over 90 generations as alcohol consumers (University of Chile Bibulous; UChB),[18,47] were used in the experiments. Animals were maintained on a 12-h light/dark cycle (lights off at 7:00 PM) and regularly fed a soy protein, peanut-meal rodent diet (Cisternas, Santiago, Chile). The UChB rats used in the present study had undergone brother–sister mating for seven generations. These animals are available for independent scientific purposes upon request. The experiments were carried out following the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health of the United States.

2.2 | Antibiotic administration prior to ethanol access and subsequent voluntary ethanol intake evaluation

To evaluate the role of *innate* gut microbiota on the acquisition phase of ethanol intake, 2-month-old UChB ethanol-naïve rats were single-housed and daily administered for 7 days an oral mix of two non-absorbable antibiotics; neomycin (250 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) and polymyxin B (9 mg/kg, Sigma-Aldrich) were dissolved in sterile water and delivered by oral gavage in a volume of 6 ml/kg/day.[48] Control rats were treated with vehicle (6 ml/kg sterile water). This antibiotic combination was chosen due to its ability to modulate the gut microenvironment, making changes in the balance of the two dominant phyla: Firmicutes and Bacteroidetes in murine models.[48] Additionally, this antibiotic combination is able to prevent liver injury in rats following long-term exposure to ethanol.[49] One day after the last antibiotic or vehicle administration, rats were allowed free-choice access to 10% (v/v) and 20% (v/v) ethanol solutions, prepared from absolute ethanol (Merck, Darmstadt, Germany), and tap water for 6 days in their own box as we previously described.[50] Ethanol and water intakes were recorded daily, and the bottle positions were alternated every day to avoid the development of side preference. Ethanol intake was expressed as g of ethanol consumed/kg body weight/day.

2.3 | Gut microbiome analysis

Twenty-four hours after the last antibiotic or vehicle administration stool samples, prior to ethanol access, were freshly collected and immediately stored at −80°C until use. DNA from feces was extracted using the QIAGEN Soil DNA extraction kit using a modified bead-beating protocol. DNA sequencing was performed by the University of Wisconsin Biotechnology Center by creating multiplexed barcoded amplicons from the 16S rRNA V4 region, and 2 × 250-bp paired-end sequencing was done using an Illumina HiSeq 2500 in rapid run mode. Raw sequencing data were curated and analyzed using high-performance computing (HPC) power resources at the UDD-Bioinformatics Center using the DADA2 pipeline.[51] In summary, all sequences were filtered to truncate the paired reads to 150 nt, and reads with quality values <2 were eliminated. Error rates were estimated and corrected by pooling all the reads from the sequencing run, with default parameters. Taxonomy was assigned using the SILVA database.[52] Resulting amplicon sequence variants (ASVs) were analyzed using the Phyloseq package in R, and chimeric sequences were removed using the Chimera Slayer algorithm.[53] For alpha diversity determinations, the number of species present in each sample group (richness) was evaluated using the Chao1 index and coupled to the abundance of the species (evenness) to calculate overall alpha diversity using Shannon's index. The code used and bioinformatics pipelines are available in the GitHub repository https://github.com/microb-r.

2.4 | Quantification of mRNA levels of proinflammatory and anti-inflammatory factors in the proximal colon and liver

Twenty-four hours after the last antibiotic or vehicle administration to ethanol-naïve rats, animals were anesthetized with chloral hydrate (280 mg/kg, i.p.) and euthanized. Proximal colon and liver samples were rapidly collected and frozen in liquid nitrogen. Total RNA was purified using Trizol (Invitrogen, Grand Island, NY). One microgram of total RNA was used to perform reverse transcription with Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) and oligo dT primers. Real-time polymerase chain reaction (RT-PCR) reactions were made using a Light-Cycler 1.5 thermocycler (Roche, Indianapolis, IN) to amplify the mRNA level of IL-6, IL-1β, TNF-α, and HO-1 using the following primers: IL-6 sense 5′-TCCTACCCC AACTTCCAATGCTC-3′, IL-6 antisense 5′-TTGGATGGTCTTGGTCC TTAGCC-3′; TNF-α sense 5′-AAATGGGCTCCCTCTCATCA TTC-3′, TNF-α antisense 5′-TCTGCTTGGTGGTTTGCTACGAC-3′; IL-1β sense 5′-CACCTCTCAAGCAGAGCACAG-3′, IL-1β antisense 5′-GGGTTCCA TGGTGAAGTCAAC-3′; HO-1 sense 5′-CTATCGTGCTCGCATGAAC-3′, HO-1 antisense 5′-CAGCTCCTCAAACAGCTCAA-3′. Additionally, in proximal colon samples, the mRNA level of the tight junction associated protein occludin was measured using the following primers: occludin sense 5′-CCCAGGTGGCAGGTAGATTA-3′, occludin antisense 5′-AGGCCTGTTTTGTGAGCACT-3′.

To ensure that amplicons were generated from mRNA and not from genomic DNA, controls without reverse transcriptase during the reverse transcription reaction were included. Relative quantifications were performed by the ΔΔCT method. The mRNA level of each target gene was normalized against the mRNA level of the housekeeping gene β-actin in the same sample.

2.5 | Immunofluorescence and colocalization analysis from rat proximal colon sections

Paraffin histological sections derived from proximal colon samples obtained from vehicle- and ABXs-treated rats were evaluated for the co-expression of occludin (Ocln) with E-cadherin by immunofluorescence. Briefly, the sections were subjected to deparaffinization (NeoClear, Merck KGaA, Darnstadt, Germany) and rehydrated with a battery of alcohols from absolute ethanol to 70° ethanol. Antigenic recovery was performed with sodium citrate buffer (pH 6). Then, the slides were incubated with 100-mM glycine and 2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in 1X phosphate-buffered saline (PBS), for autofluorescence and nonspecific proteins blocking, respectively. The slides were incubated overnight at 4°C with the following primary antibodies anti-Ocln (1/50; polyclonal rabbit antibody, cat. N° 71-1500, Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) in conjunction with anti-E-cadherin (E-cad) (1/300; monoclonal mouse antibody, cat. N° 610181, BD Biosciences, Franklin Lakes, NJ, USA). After PBS rinse, tissue sections were incubated for 1 h at room temperature with secondary antibodies (Thermo-Fisher Scientific, Waltham, MA, USA), Donkey Anti-Rabbit IgG conjugated with Alexa Fluor 647 (1/200) and Donkey Anti-Mouse IgG conjugated with Alexa Fluor 488 (1/200). Hoechst 33342 (1/500) was used as a nuclear counterstain. Finally, slides were coverslip-covered with Dako™ mounting medium (Dako, Agilent Technologies Inc., Santa Clara, CA, USA). Slides were visualized by Olympus Fluoview F10i confocal microscope at 10X and 60X objectives (Nikon Instruments Inc, Melville, NY, USA).

Colocalization analysis was performed with the FIJI version of Image J software (http://fiji.sc/) using the Coloc2 plugin only on epithelium selected as the region of interest (ROI). Pearson correlation coefficient was used to compare the intensity correlation between Occludin with E-cadherin channels among vehicle- and ABXs-treated animals.

2.6 | Determination of LPS levels in portal blood samples and cytokine gene expression in liver

Twenty-four hours after the last antibiotic or vehicle administration to ethanol-naïve rats, animals were anesthetized with chloral hydrate (280 mg/kg, i.p.), and blood samples were collected by portal vein puncture. LPS levels were determined in plasma using the rat LPS enzyme-linked immunosorbent assay (ELISA) kit (MyBiosource, CA) following the manufacturer's instructions. Liver cytokine mRNA levels were determined as described above in liver samples collected at the same time.

2.7 | LGG probiotic treatment

To evaluate the role of probiotic supplementation on ethanol intake, 2-month-old UChB ethanol-naïve rats were single-housed, allowed the standard diet and water, and administered for 13 consecutive days a daily slurry of 5×10^9 colony forming unit (CFU) LGG (Vivera ®, Probiotica S.p.A; Novara, Italy) suspended in 1 ml of PBS, pH 7.2 or vehicle (1 ml of PBS solution) by oral gavage.[54] Following the initial 8 days of LGG administration, rats were allowed concurrent two-bottle choice access to 10% and 20% (v/v) ethanol solution and water, for 5 days. Ethanol intake was recorded daily and expressed as g of ethanol consumed/kg body weight/day. Twenty-four hours after the alcohol consumption assessment period, rats were anesthetized with chloral hydrate (280 mg/kg, i.p.), and blood samples (1.5 ml) were collected by cardiac puncture, for plasma FGF21 determination. Thereafter, rats were euthanized, and liver samples were collected for FGF21 mRNA determinations. Plasma FGF21 was analyzed by the rat FGF21 Quantikine ELISA kit (R&DSystems, MN) following the manufacturer's instructions. For hepatic FGF21 mRNA determinations, total RNA was purified from liver samples using TRIzol (Invitrogen, Grand Island, NY). One microgram of total RNA was used to perform reverse transcription with MMLV reverse transcriptase (Invitrogen) and oligo dT primers. Real-time PCR reactions were made using a Light-Cycler 1.5 thermocycler (Roche, Indianapolis, IN) to amplify the mRNA level

of FGF21 using the following primers FGF21 sense 5′-CAAATCCTGG GTGTCAAAGC-3′; FGF21 antisense 5′-GCCTCAGACTGGTACA CATTG-3′. To ensure that amplicons were generated from mRNA and not from genomic DNA, controls without reverse transcriptase during the reverse transcription reaction were included. Relative quantifications were performed by the ΔΔCT method. The mRNA level of FGF21 was normalized against the mRNA levels of the housekeeping gene β-actin in the same sample.

2.8 | Subdiaphragmatic vagotomy and subsequent access to ethanol

To evaluate the role of the vagus nerve on the acquisition phase of ethanol intake, 2-month-old UChB ethanol-naïve rats underwent subdiaphragmatic vagotomy as previously described.[55] Briefly, animals were anesthetized with ketamine (70 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and the stomach and lower esophagus were visualized after upper midline laparotomy. Within a 3-mm radius around the incision site, the skin layer was separated from the muscular layer using blunt dissecting scissors, and the intestine was retracted to allow access to the liver and the stomach. The left lateral lobe of the liver was then retracted to visualize the distal end of the esophagus. A ligature was placed around the esophagus at its entrance to the stomach to allow gentle retraction to clearly expose both vagal branches. Using jeweler's forceps, both branches of the vagus nerve were isolated from the esophagus, and a 1- to 2-mm section of each branch of the nerve was resected with scissors. The second group of sham-operated rats, in which vagal branches were similarly exposed but not cut, was included as a control. In both groups, the incisions were closed following surgery. All animals were kept under special postsurgical care for 3 days, and a 13-day recovery period was allowed before alcohol preference was tested. There was no mortality or differential weight changes in vagotomized and sham-operated animals. Two weeks after surgery, rats received continuous free-choice access to 10% and 20% (v/v) ethanol solutions and tap water for 6 days. Ethanol intake was recorded daily and expressed as g of ethanol consumed/kg body weight/day. Total fluid intake was also recorded daily.

2.9 | Vagotomy verification

The efficacy of vagotomy was assessed using a rectal temperature test, based on the febrile effect induced by LPS, mediated by the vagus nerve.[55] The test was performed 48 h after the alcohol consumption assessment period had ended; the initial rectal temperature was measured in vagotomized and sham-operated rats, using a 0.125-inch-diameter probe for adult rats with a Thermalert Monitoring Thermometer (Physitemp, Clifton, NJ). Thereafter, sham and vagotomized rats were intraperitoneally injected 200 μg/kg LPS (Sigma-Aldrich) dissolved in saline and delivered as 5.0 ml/kg. Rectal temperature was determined 24 h after LPS administration.

2.10 | Saccharin intake in antibiotic-treated or vagotomized rats

To evaluate the effect of antibiotic administration on saccharin intake in naïve UChB rats, the nonabsorbable antibiotic mix neomycin (250 mg/kg, Sigma-Aldrich) and polymyxin B (9 mg/kg, Sigma-Aldrich) was administered by oral gavage dissolved in sterile water daily for 7 days in a volume of 6 ml/kg/day. Control rats were treated with vehicle (6 ml/kg sterile water). One day after the last antibiotic or vehicle administration, rats were exposed to the choice between 0.3% (w/v) saccharin solution or water,[56] for 6 days. To evaluate the effect of vagotomy on saccharin intake, naïve UChB rats underwent subdiaphragmatic vagotomy or sham surgery as described above, and 14 days after surgery, the animals were allowed access to the 0.3% (w/v) saccharin solution or water for 6 days. The saccharin solution was prepared by dissolving saccharin (Sigma-Aldrich) in distilled water. Saccharin intake was recorded daily and expressed as ml of saccharin consumed/kg body weight/day.

2.11 | Statistical analysis

Statistical analyses were performed using GraphPad Prism (San Diego, CA, USA). Data are expressed as means ± SEM. The normal distribution of data for all experiments was first tested using the Shapiro–Wilk test. For normal distributed data, one-way or two-way analysis of variance (ANOVA) was used followed by a Tukey or Fisher post hoc test. When only two groups were compared, statistical significance was determined by Student's *t* test. A level of $P < .05$ was considered for statistical significance. To facilitate text reading, full statistical ANOVA analyses are presented in the figure legends.

2.12 | Experimental design

A figure that depicts the sequence and type of studies conducted is included as Figure S1.

3 | RESULTS

3.1 | Antibiotic administration to ethanol-naïve rats greatly reduced subsequent ethanol intake

To determine whether the innate gut microbiota of UChB rats influences alcohol intake, ethanol-naïve male rats were administered a mix of two nonabsorbable antibiotics (ABXs neomycin 250 mg/kg/day and polymyxin B 9 mg/kg/day), which has been previously shown to alter the gut microbiota of rats[48] by oral gavage for 7 days. Voluntary alcohol preference was subsequently evaluated daily for 6 days, starting 1 day after antibiotic discontinuation. Animals were offered a three-bottle fluid choice, namely, access to 10% v/v ethanol, 20% v/v ethanol, and water (first 24-h intake shown as Day 9). Antibiotic

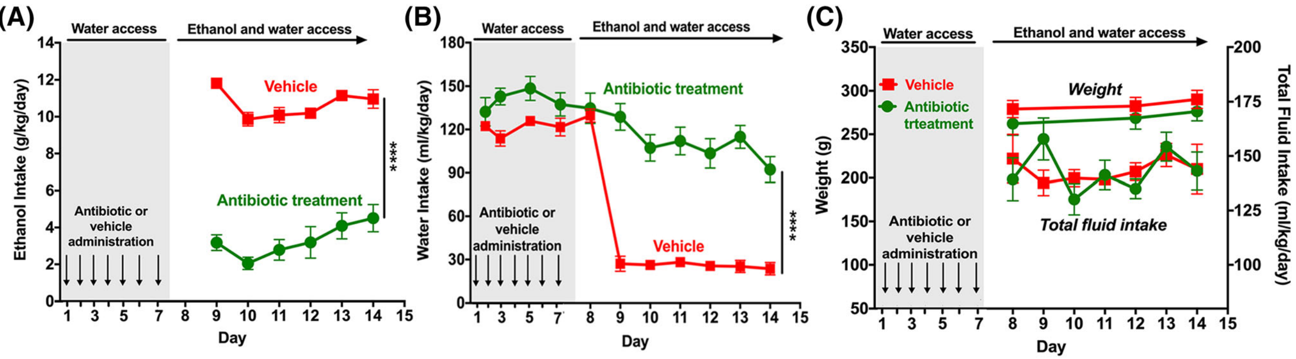

FIGURE 1 Oral antibiotic administration before ethanol access inhibits voluntary ethanol intake. A, Voluntary ethanol intake of rats pretreated (shaded area) daily with a mix of two nonabsorbable antibiotics (ABXs: neomycin 250 mg/kg/day and polymyxin B 9 mg/kg/day) or vehicle for 7 days. Arrows indicate antibiotic or vehicle administration. Two-way analysis of variance (ANOVA) (treatment × day) of ethanol intake following administration (green dots) showed a significant effect of treatment (****$P < .0001$), compared with control rats receiving vehicle (red squares) and significant effect of day (**$P < .0022$), while there was not significant treatment × day interaction. Bonferroni post hoc analysis revealed that antibiotic treatment inhibited ethanol intake (65%) during the 6 days recorded versus vehicle-treated control (****$P < .0001$; $n = 6$ per group). B, Voluntary water intake of animals pretreated daily with two nonabsorbable antibiotics (neomycin 250 mg/kg/day and polymyxin B 9 mg/kg/day) or vehicle orally for 7 days. Two-way ANOVA (treatment × day) of water intake following antibiotic administration (green dots) indicates significant effect of treatment (****$P < .0001$), compared with control rats receiving vehicle (red squares), but not significant effect of day. Bonferroni post hoc analysis revealed that antibiotic treatment increases water intake (400%) during the 7 days recorded versus vehicle-treated control (****$P < .0001$; $n = 6$ per group). C, Total fluid intake and body weight were not affected by antibiotic treatment. All data are presented as mean ± SEM

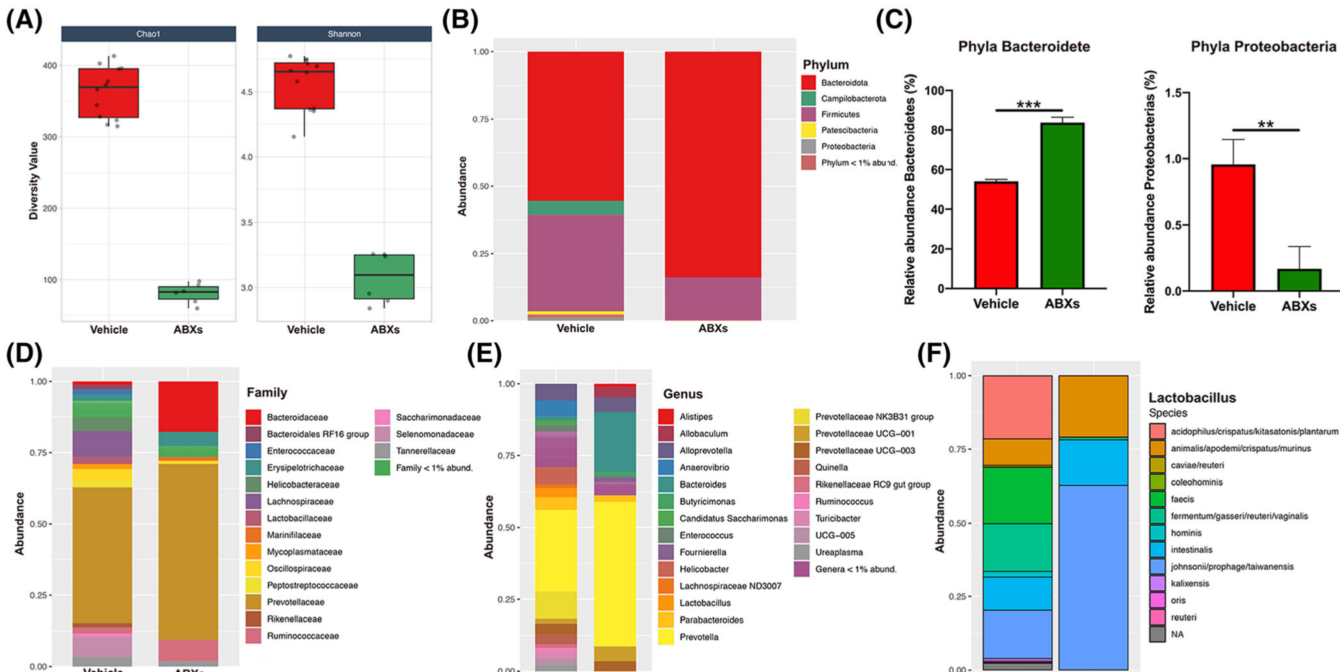

FIGURE 2 Oral antibiotic administration significantly alters the composition of the gut microbiota. A, Chao-1 and Shannon diversity indices (α-diversity) of ethanol-naïve animals that were daily administered the nonabsorbable antibiotics (ABXs: neomycin 250 mg/kg/day and polymyxin B 9 mg/kg/day) or vehicle orally for 7 days. A marked reduction in gut microbiota diversity is seen in the antibiotic-treated rats. B, Relative abundance at the phylum level. C, Relative increase in abundance of the phyla Bacteroidetes (***$P < .001$) and marked reduction of Proteobacteria (**$P < .01$) are seen in antibiotic-treated animals. D, Relative abundance at the family level. E, Relative abundance at the genus level. F, Relative abundance of species of *Lactobacillus* of ethanol-naïve animals treated with antibiotics or vehicle for 7 days. A fivefold increase in *Lactobacillus johnsonii* is seen ($n = 6$–12 per group)

administration reduced by 70% ($P < .0001$), the overall voluntary ethanol intake compared with that of vehicle-treated controls (Figure 1A). The inhibition of ethanol intake induced by ABXs administration was compensated by an increase in water intake ($P < .001$) (Figure 1B), normalizing the animal's hydric homeostasis (Figure 1C). Animal growth, determined by weight gain during the treatment period, was not affected by ABXs administration (Figure 1C).

3.2 | Oral antibiotic administration significantly alters the composition of gut microbiota

As oral antibiotic administration dramatically reduced the gut microbiome load,[57] a 16S rRNA sequencing was applied to detect the microbiota profile in feces collected from ethanol-naïve animals 24 h after the last 7-day antibiotic or vehicle administration. Initially, we analyzed the richness of the gut community (alpha diversity), and as expected, the gut microbiota of antibiotics-treated UChB rats (ABXs) showed a significant reduction in the Chao-1 diversity index (number of unique species) and Shannon diversity index (richness and evenness of bacterial community) (Figure 2A). Subsequently, we analyzed the composition of the gut microbiota at the phylum level, which showed that the Bacteroidetes and Firmicutes were the main community members, both in vehicle and ABXs-treated animals (Figure 2B). Interestingly, ABXs administration greatly reduced the relative abundance of Proteobacteria, a highly proinflammatory phylum previously associated with high alcohol intake[58] and significantly increases the relative abundance of phylum Bacteroidetes; a reduction in this phylum has been previously associated with high alcohol intake (Figure 2C).[58] The relative abundance of gut microbiota at the family (Figure 2D) and genera levels (Figure 2E) also showed differences between ABXs- and vehicle-treated animals. Additionally, we analyzed the relative abundance of *Lactobacillus* at the species level because some *Lactobacillus* species have potent anti-inflammatory properties. We observed that ABXs treatment induced an important increase in the relative abundance of the anti-inflammatory *Lactobacillus johnsonii* (Figure 2F) compared with vehicle-treated animals, possibly protecting the intestinal lumen.[59]

3.3 | Oral antibiotic administration reduces intestinal inflammation while increasing the tight junction protein level

Gut dysbiosis is associated with the development of intestinal inflammation and the concomitant reduction in the expression levels of different tight junction components, resulting in an increased gut permeability that facilitates the translocation of intestinal microbiota components to the portal circulation.[26,60] To evaluate whether antibiotic administration could reduce intestinal inflammation, samples from the proximal colon were obtained 24 h after the last antibiotic or vehicle administration, while mRNA levels of proinflammatory and anti-inflammatory factors were determined by RT-qPCR. Antibiotic administration reduced the intestinal mRNA levels of the main proinflammatory cytokines, namely, IL-6, IL-1β, and TNF-α (Figure 3A–C), while the mRNA level of Heme Oxygenase-1 (HO-1), an enzyme with potent anti-inflammatory properties,[61] was increased by the antibiotic treatment (Figure 3D).

As expected, the reduction in colonic proinflammatory cytokine mRNA levels induced by the antibiotic administration associates with increased tight junction occludin at mRNA level (Figure 4A), and protein level, specifically located in the epithelial cell plasma membrane (colocalization with E-cadherin) compared with vehicle-treated animals (Figure 4B,C). Nevertheless, the increased proinflammatory cytokine and reduced occludin levels observed in gut in vehicle-treated animals were not associated with increased intestinal LPS

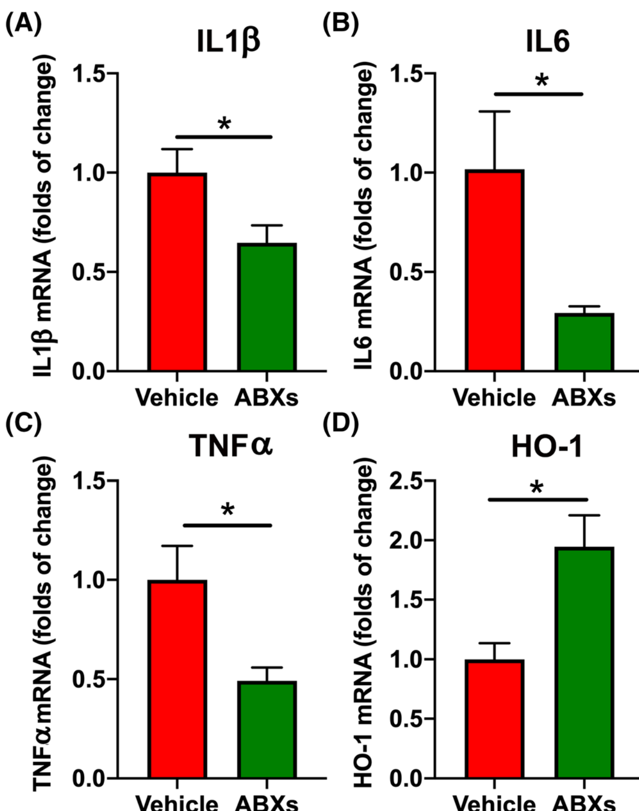

FIGURE 3 Oral antibiotic administration reduces intestinal inflammation. mRNA levels of the proinflammatory cytokines A, IL-6, B, IL-1β, C, TNF-α, and D, the anti-inflammatory enzyme Heme Oxygenase-1 (HO-1), determined by real-time quantitative polymerase chain reaction (RT-qPCR) in samples of the proximal colon of ethanol-naïve animals that were daily administered the nonabsorbable antibiotics (ABXs: neomycin 250 mg/kg/day and polymyxin B 9 mg/kg/day) or vehicle orally for 7 days. Animals were euthanized and tissue collected 1 day after the last antibiotic or vehicle administration; mRNA levels of target genes were normalized against the expression of the housekeeping gene β-actin and expressed as fold change compared with values for vehicle-treated animals (two-tailed *t* test, $^*P < .05$; $n = 6$ per group). A significant reduction in the mRNA levels of proinflammatory cytokines and a significant increase in the mRNA level of the anti-inflammatory HO-1 enzyme were observed in antibiotic-treated animals. All data are presented as mean ± SEM

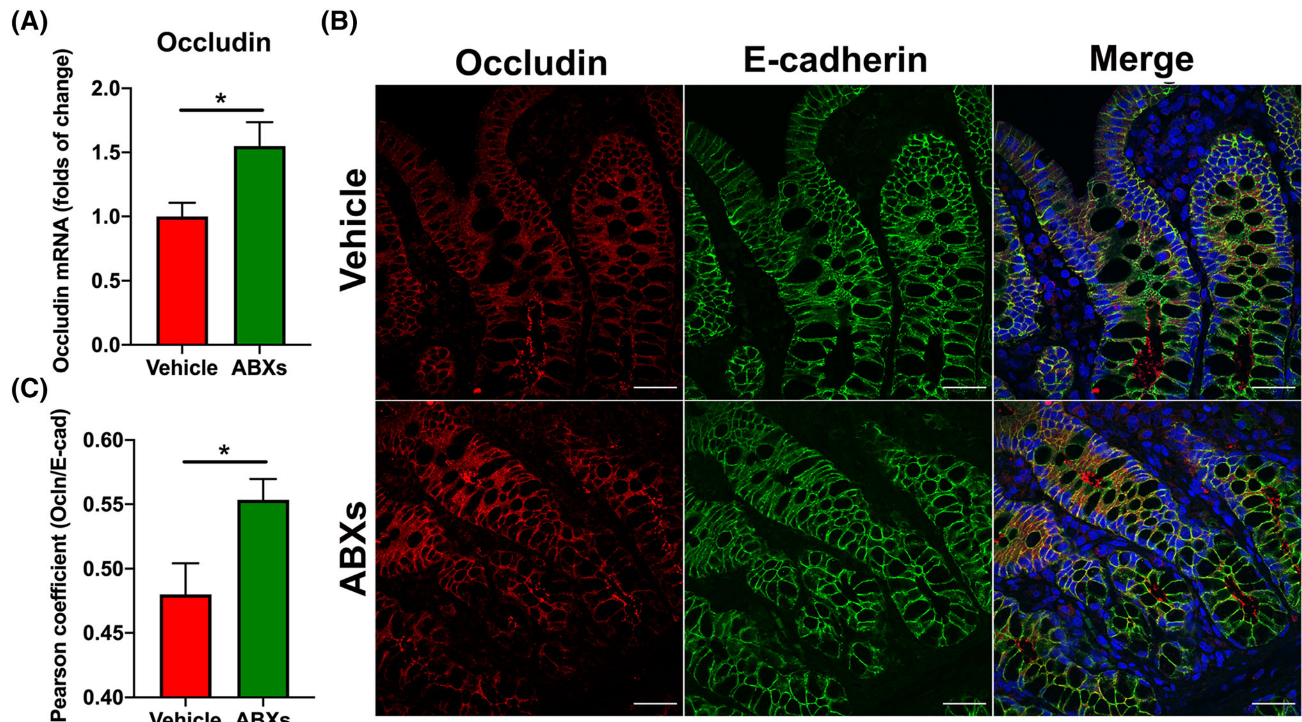

FIGURE 4 Oral antibiotic administration increases occluding levels in the proximal colon. A, mRNA level of occludin determined by real-time quantitative polymerase chain reaction (RT-qPCR) in proximal colon samples of ethanol-naïve animals that were daily administered the nonabsorbable antibiotics (ABXs: neomycin 250 mg/kg/day and polymyxin B 9 mg/kg/day) or vehicle orally for 7 days, 1 day after antibiotic or vehicle discontinuation. Occludin mRNA level was normalized against the expression of the housekeeping gene β-actin and expressed as fold change compared with vehicle-treated animals. A significant increase in occludin mRNA was observed in antibiotic-treated animals (two-tailed *t* test, $^*P < .03$; $n = 6$). B, Representative confocal microphotographs of proximal colon samples showing colocalization of occludin immunoreactivity (red staining) with the plasmatic membrane marker E-cadherin (green staining). Nuclei were counterstained with Hoechst (blue staining). Scale bar 30 μm. C, Pearson correlation coefficient (occludin/E-cadherin) showing a significant increase in the presence of occludin in the plasma membrane of intestinal epithelial cells of antibiotic-treated animals compared with vehicle-treated animals (two-tailed *t* test, $^*P < .03$; $n = 6$). All data are presented as mean ± SEM

permeability, evaluated by ELISA in portal blood (Figure S2A), nor was with the synthesis of liver proinflammatory cytokines evaluated by RT-qPCR (Figure S2B–D), suggesting that the dysbiotic microbiota generates a gut-localized rather than a systemic inflammation in UChB rats (likely being transferred to the brain via the vagus nerve; *vide infra*).

3.4 | Oral LGG administration increases FGF21 levels and reduces voluntary ethanol intake

Probiotic supplementation is a noninvasive approach to alter gut microbiota composition. Among different probiotics, the gram-positive LGG can generate an anti-inflammatory microenvironment in the intestinal lumen, and its level is reduced in animals exposed to alcohol and in alcohol-dependent patients.[62]

To evaluate if such a probiotic supplementation could reduce alcohol intake, alcohol-naïve UChB rats were administered LGG daily (5×10^9 each dose) or vehicle orally for 13 days. After the eighth administration of LGG, daily voluntary ethanol intake was evaluated for 5 days using the three-bottle choice paradigm (10% and 20% v/v ethanol solutions and water). LGG supplementation induced a 35% reduction of voluntary alcohol intake ($P < .01$) versus that in vehicle-treated rats (Figure 5A), and conversely a marked increase in water intake ($P < .001$) (Figure 5B). The present study further confirmed that LGG administration leads to a pronounced increase in plasmatic FGF21 levels ($P < .01$) (Figure 5C) evaluated by ELISA and hepatic mRNA FGF21 levels ($P < .01$) (Figure 5D) evaluated by RT-qPCR compared with vehicle-treated rats. Concordantly, LGG supplementation has been reported to have a therapeutic effect in an animal model of inflammatory fatty liver disease.[46]

3.5 | Subdiaphragmatic vagotomy prior to ethanol access inhibits voluntary ethanol intake

The vagus nerve is the neural axis between the gut (including gut bacteria) and the brain and also an important route for the translation of peripheral inflammation to the brain.[6,10,64] To evaluate the role of the vagus nerve on the acquisition of high ethanol intake, ethanol-naïve UChB rats were subjected to a subdiaphragmatic vagotomy before allowing their access to ethanol (10% and 20% v/v) and water for an

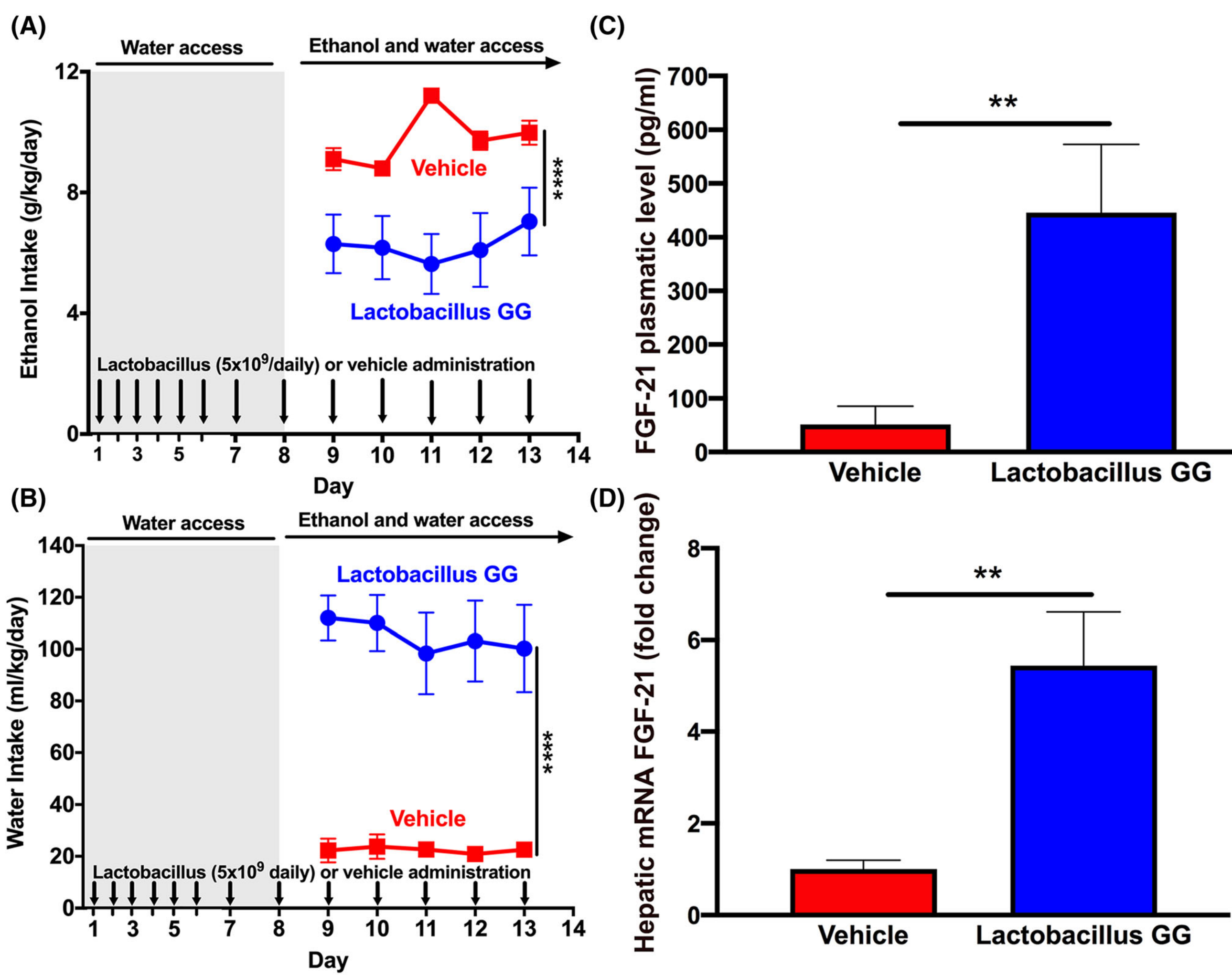

FIGURE 5 Oral *Lactobacillus* Gorbach–Goldin (GG) administration reduces voluntary ethanol intake and increases fibroblast growth factor-21 (FGF21) levels. A, Voluntary ethanol intake of rats that were treated with a daily oral administration of *Lactobacillus* GG (5×10^9 daily) or vehicle for 13 days. On Day 8, access to ethanol solutions (10% and 20%) and water was allowed, and ethanol intake was determined daily for 5 days. Arrows indicate *Lactobacillus* GG or vehicle administration. Two-way analysis of variance (ANOVA) (treatment × day) of ethanol intake following *Lactobacillus* GG administration (blue dots) indicates a significant effect of treatment (****$P < .0001$), compared with control animals receiving vehicle (red squares), but not significant effect of day. Bonferroni post hoc analysis revealed that overall *Lactobacillus* treatment inhibited ethanol intake (35%) during the 5 days recorded versus vehicle-treated control (**$P < .01$; $n = 6$ per group). B, Voluntary water intake of ethanol-naïve animals that were treated with daily oral administration of *Lactobacillus* GG (5×10^9 daily) or vehicle for 13 days. Two-way ANOVA (treatment × day) of water intake following *Lactobacillus* GG administration (blue dots) indicates significant effect of treatment (****$P < .0001$), compared with control animals receiving vehicle (red squares), but not significant effect of day. Bonferroni post hoc analysis revealed that *Lactobacillus* GG administration increases water intake (400%) during the 5 days recorded versus vehicle-treated control (****$P < .0001$; $n = 6$ per group). It is noted that FGF21 has per se an added diuretic effect.[63] C, Plasma FGF21 levels determined by enzyme-linked immunosorbent assay (ELISA) in blood samples of animals orally administered *Lactobacillus* GG (blue bar) for 13 days increased 11-fold versus those in vehicle-treated animals (red bar) (two-tailed *t* test, **$P < .01$; $n = 6$ per group). D, Marked increases in hepatic mRNA levels of FGF21 determined by real-time quantitative polymerase chain reaction (RT-qPCR) in liver samples of animals treated by 13 daily oral administrations of *Lactobacillus* GG (blue bar) versus those in vehicle-treated animals (red bar). FGF21 mRNA levels were normalized against the expression of the housekeeping gene β-actin and expressed as fold change compared with vehicle-treated animals (two-tailed *t* test, **$P < .01$; $n = 6$ per group). All data are presented as mean ± SEM

ethanol intake evaluation. Figure 6A shows that subdiaphragmatic vagotomy led to an overall 80% reduction ($P < .001$) in voluntary ethanol intake compared with sham-operated animals. As expected, this reduction of ethanol intake induced by the vagotomy was compensated by the increase in water intake ($P < .001$) (Figure 6B), suggesting a clear role of the vagus nerve on the acquisition phase of alcohol intake, rather than on fluid intake.

The efficacy of vagotomy was assessed in the above animals by a rectal temperature test, based on the febrile effect induced by LPS, which is mediated by the vagus nerve.[55] Twenty-four hours after the alcohol consumption period had ended, baseline rectal temperature in sham and vagotomized rats was measured, and thereafter, animals were administered LPS intraperitoneally. Rectal temperature was measured again 24 h after LPS administration. As expected, the LPS

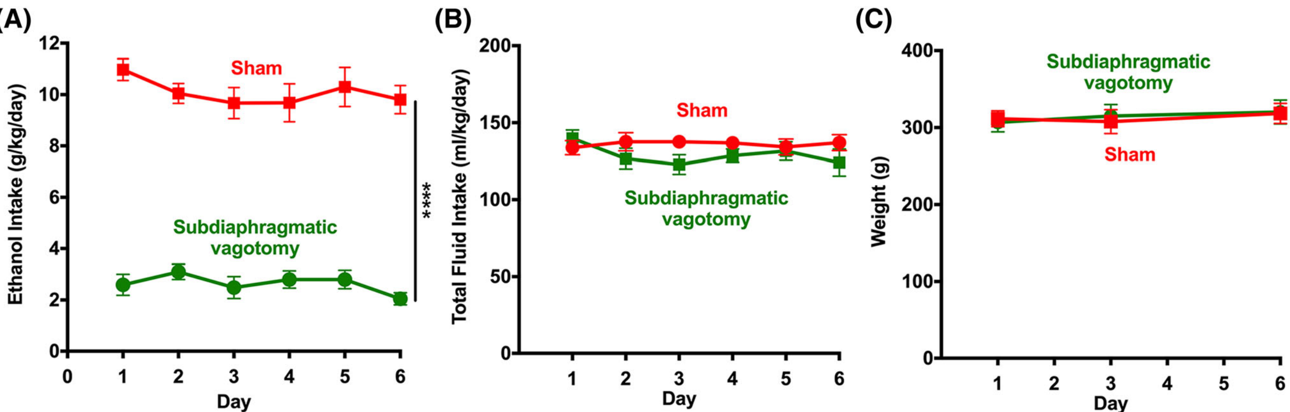

FIGURE 6 Subdiaphragmatic vagotomy prior to ethanol access markedly inhibits voluntary ethanol intake. A, Voluntary ethanol intake of animals that was subjected to a subdiaphragmatic vagotomy or a sham surgery 2 weeks before ethanol access. Two-way analysis of variance (ANOVA) (treatment × day) of ethanol intake in subdiaphragmatic vagotomy animals (green dots) indicates a significant effect of treatment (****$P < .0001$), compared with ethanol intake by sham operated animals (red squares), but not significant effect of day. Bonferroni post hoc analysis indicates that subdiaphragmatic vagotomy inhibited ethanol intake (80%) versus that in sham animals during the 6 days recorded (****$P < .0001$; $n = 6$ per group). B, Total fluid intake of animals that were subjected to subdiaphragmatic vagotomy was not significantly different from that of sham-operated animals. Increases in water intake in vagotomized animals compensated for their reduced fluid intake from alcohol solutions. C, Body weight of animals after vagotomy. Vagotomy did not affect body weight. All data are presented as mean ± SEM

administration induced an increase in body temperature in sham-operated rats, an effect that was completely absent in vagotomized animals (Figure S3).

3.6 | Saccharin intake by the high ethanol drinker UChB rats is markedly inhibited by antibiotic pretreatment or vagotomy

A strong association exists between the ingestion of ethanol and sweet-tasting solutions. Enhanced consumption of sweet solutions is one of the most consistent phenotypic predictors of alcohol intake common across alcohol-preferring rodent strains.[65,66] Similarly, alcoholics prefer more highly concentrated sucrose solutions than non-alcoholic control subjects.[35,67] Thus, we evaluated if antibiotic administration or vagotomy, in addition to preventing alcohol intake, could also reduce saccharin intake. Figure 7A shows that a prior 7-day antibiotic administration to rats inhibited saccharin intake by an overall 87% ($P < .0001$), compared with the saccharin intake of vehicle-treated rats. It is noted that antibiotic administration reduced saccharin intake despite the marked polydipsia known to characterize the pharmacologically unimpeded high intake of saccharin, which led to an increase in total fluid and saccharin intake (Figure 7B). Similarly, Figure 7C shows that prior vagotomy inhibited by 75% ($P < .0001$) the voluntary intake of saccharin compared with that of sham-operated animals. A total increase in fluid intake was also observed for sham-operated saccharin drinking animals (Figure 7D). The marked reduction of saccharin intake achieved by antibiotic administration and vagotomy, as also seen for ethanol intake, strengthens the view that the preference for sweet flavors and ethanol shares common mechanisms.[56]

4 | DISCUSSION

Data presented indicate that innate gut bacteria strongly influences voluntary alcohol consumption in rats selectively bred for their high ethanol intake. The administration of two nonabsorbable antibiotics *prior to allowing ethanol access* markedly inhibited (70%) voluntary ethanol intake, an effect fully observed on the first day of ethanol access.

The above observation is noteworthy because chronic alcohol intake has per se been reported to generate gut microbial derangements (dysbiosis), possibly potentiating alcohol intake. Leclercq et al.[26] investigated whether changes in gut permeability were linked to the gut-microbiota composition in alcohol-dependent subjects. The study found that despite similar amounts of alcohol consumed, some *but not all* alcohol-dependent subjects developed gut leakiness and an altered microbiota phenotype, which after 3 weeks of abstinence was associated with higher scores of alcohol craving. These findings suggest either that specific gut microbial may exist before the development of the AUD in some susceptible patients or it may take longer than 3 weeks of abstinence for ethanol-altered microbiota to return to baseline.

Recent animal studies showed that the transplantation of fecal bacteria from alcoholic patients into C57BL/6J mice, whose gut microbiota had been suppressed by antibiotic pretreatment, significantly increased their alcohol preference compared with that of mice transplanted with feces of control subjects who had not ingested alcohol for 1 year.[16] While there were many fecal bacterial differences in alcoholics and controls, the most significant difference reported was a twofold increase in the relative abundance of highly proinflammatory Proteobacteria in the alcoholic group versus controls. Whether in the alcoholics the greater abundance of Proteobacteria followed or preceded their AUD, it is not clear. In the present study, prior to ethanol

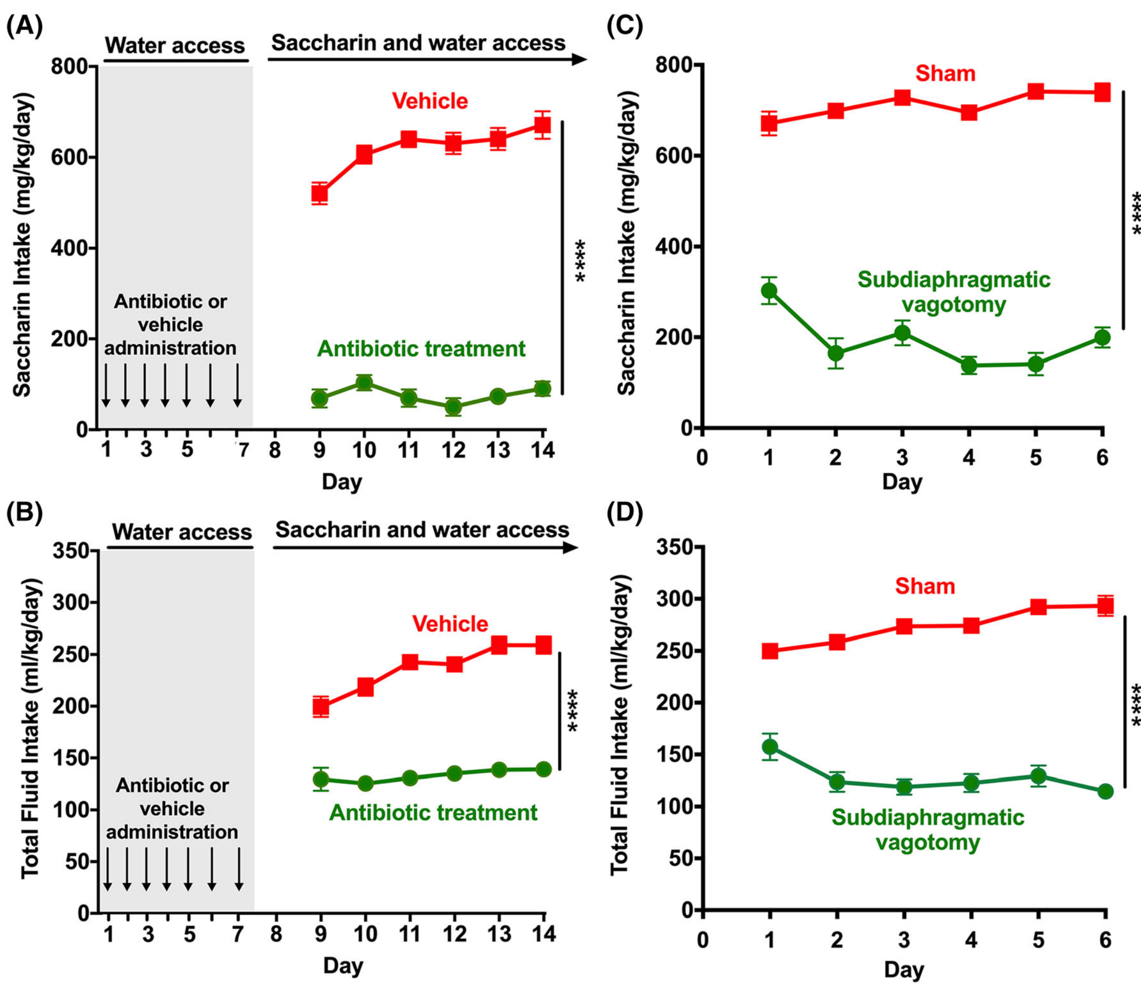

FIGURE 7 Both oral antibiotic administration and vagotomy markedly inhibit saccharin intake. A, Saccharin intake of rats pretreated (shaded area) daily with a mix of nonabsorbable antibiotics (ABXs: neomycin 250 mg/kg/day and polymyxin B 9 mg/kg/day) or vehicle for 7 days. Arrows indicate antibiotic or vehicle administration. Two-way analysis of variance (ANOVA) (treatment × day) of saccharin intake data following antibiotic administration (green dots) compared with those of control rats receiving vehicle (red squares) showed a significant effect of treatment (****$P < .0001$), significant effect of day (**$P < .005$), and a significant treatment × day interaction (*$P < .05$). Bonferroni post hoc analysis revealed that antibiotic treatment inhibited saccharin intake (87%) during the 6 days recorded versus vehicle-treated control (****$P < .0001$; $n = 6$ per group). B, Total fluid intake of animals pretreated daily with two nonabsorbable antibiotics or vehicle orally for 7 days. Two-way ANOVA (treatment × day) of total fluid intake data indicates significant effect of treatment (****$P < .0001$) but not significant effect of day. Bonferroni post hoc analysis revealed that the antibiotic-treated animals displayed a significantly lower total fluid intake (green dots) compared with control rats receiving vehicle (red squares). C, Voluntary saccharin intake of rats subjected to subdiaphragmatic vagotomy or to sham surgery. Two-way ANOVA (treatment × day) of saccharin intake data indicates significant effect of treatment (****$P < .0001$), significant effect of day (*$P < .05$), and a significant treatment × day interaction (***$P < .001$). Bonferroni post hoc analysis indicates that subdiaphragmatic vagotomy (green dots) inhibited saccharin intake (73%) versus sham-operated animals (red squares), during the 7 days recorded (****$P < .0001$; $n = 6$ per group). D, Total fluid intake of animals subjected to subdiagramatic vagotomy or to sham surgery. Two-way ANOVA (treatment × day) of total fluid intake following vagotomy (green dots) compared with control rats receiving vehicle (red squares) indicates a significant effect of treatment (****$P < .0001$) but not significant effect of day. All data are presented as mean ± SEM

exposure, the vehicle-treated (control) high-drinker UChB rats showed a fourfold greater abundance of gut Proteobacteria, versus antibiotic-treated UChB rats. It is noted that both in alcoholics and in alcohol-naïve UChB rats, the relative abundance of Proteobacteria was high, although only 2%; thus, these may be representative of a general type of proinflammatory phyla.

As indicated above, an evolution-derived fail-safe mechanism appears to exist to prevent both alcohol intoxication and the intake of sweet products. Oral alcohol and sugar intake have been shown to markedly increase FGF21 plasma levels, while the administration of recombinant FGF21 greatly inhibits the self-administration of both alcohol and saccharin.[9,41,42] In the present study, gut microbiota modification by the administration of LGG led to an 11-fold increase in plasma FGF21 levels. Despite such a large increase in FGF21, LGG administration only led to a 35% reduction of ethanol intake, suggesting the existence of a mechanism (nonsystemic) opposing the

FGF21-induced regulation of the rewarding effect of ethanol. Such a nonsystemic mechanism may be the marked colonic inflammation and vagal activation. The mechanism by which FGF21 reduces ethanol intake has been described as an activation of dopamine transporter (DAT) transcription in nucleus accumbens,[9] thus allowing less dopamine to access the postsynaptic receptor and likely reducing the rewarding effects of ethanol. Constituting an opposing effect, a vagal activation per se is reported to reduce DAT levels,[68] and recent studies indicate that animals will self-administer vagal electrical pulses,[69] in line with a rewarding (or pro-rewarding) effect of a vagal activation. Indeed, the present study showed that vagotomy inhibited by 70%–80% the alcohol intake of UChB rats.

A question that arises is the likely existence of an innate mechanism in rats selectively bred for their high ethanol intake that would allow more dopamine to be released in nucleus accumbens (thus making ethanol more rewarding, i.e., a cocaine-like effect). Noteworthy, microdialysis studies show that following a small dose of ethanol, both the Indianapolis alcohol-preferring rat (P) and the high drinker UChB rat release a greater percentage of their endogenous dopamine than their nondrinker counterparts (NP and UChA rats).[43,44]

Stimulation of the vagal nerve, leading to a reduction in striatal DAT,[68] is expected to increase the rewarding effects not only for ethanol but also for a sweet substance like saccharin because the intake of both ethanol and sweet solutions is known to release dopamine in nucleus accumbens.[43,45] In the present study, both the intakes of ethanol and saccharin were nearly suppressed by vagotomy and by oral antibiotic pretreatment. Whether the inflammatory innate gut microbiota of the high-drinker UChB plays a role akin to electrical vagal stimulation is not clear. However, present studies showed that gut proinflammatory cytokine mRNA levels were reduced by antibiotic treatment, indicating the existence of a microbiota-induced gut inflammation in ethanol-naïve animals. Thus, in ethanol-naïve animals, vagal activation is likely mediated by gut proinflammatory cytokines (e.g., IL-1β).[70] Unexpectedly, neither systemic LPS nor proinflammatory liver cytokine transcription levels were altered in ethanol-naïve UChB rats.

The study strongly indicates that the innate gut microbiota of high ethanol drinker UChB rats constitutes the main factor allowing their high alcohol intake, as the oral administration of a nonabsorbable antibiotic mix inhibited by two thirds their voluntary ethanol intake. Additionally, oral antibiotic administration suppressed gut inflammation, suggesting that gut microbiota-induced inflammation is involved in the early rewarding effects of alcohol.

It is of interest that while studies in the past decade have shown that brain inflammation is highly associated with the perpetuation of alcohol intake and relapse, present studies suggest that gut inflammation contributes to the initiation of alcohol intake. Overall, data presented indicate that the firewall mechanisms that normally prevent a high alcohol intake are suppressed by the endogenous microbiota of a rat strain selected for their alcohol preference. Further studies are necessary to determine whether innate microbiota plays a role in the development or perpetuation of human AUDs.

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AUTHOR CONTRIBUTIONS

FE contributed to the conception and design, collection of data, data analysis, financial support, manuscript writing, and final approval of the manuscript. MEQ did the conception and design, collection of data, data analysis, and final approval of the manuscript. FM-F, PM, BO, and GL did the collection of data, data analysis, and final approval of the manuscript. JMM contributed to the data analysis, financial support, and final approval of the manuscript. MAH did the design, data analysis, and final approval of the manuscript. ME did the data analysis and final approval of the manuscript. MH-M did the conception and final approval of the manuscript. YI contributed to the conception and design, data analysis, financial support, manuscript writing, and final approval of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. UChB breeding couples are available upon request for independent scientific studies.

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SUPPORTING INFORMATION

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

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