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A dual treatment blocks alcohol binge-drinking relapse: Microbiota as a new player

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ABSTRACT

Rationale: Gut microbiota communicates information to the brain. Some animals are born with a gut microbiota that predisposes to high alcohol consumption, and transplantation of fecal material from alcoholics to mice increases animal preference for ethanol. Alcohol-use-disorders are chronic conditions where relapse is the hallmark. A predictive animal model of relapse is the "alcohol deprivation effect" where ethanol re-access is allowed following chronic alcohol intake and a long alcohol deprivation. The present study evaluates the effect of gut microbiota modification on relapse, as an adjunct to N-acetylcysteine + Acetylsalicylic acid administration, which inhibits the alcohol-induced hyper-glutamatergic condition.

Methods: Rats bred as heavy alcohol consumers (UChB) were allowed ethanol intake for one month, were deprived of alcohol for two-weeks and subsequently offered re-access to ethanol. Prior to ethanol re-access animals received orally either (i) vehicle-control, (ii) L*actobacillus-rhamnosus*-GG after antibiotic treatment (LGG); (iii) N-acetylcysteine+Acetylsalicylic acid (NAC/ASA) or (iv) both treatments: LGG+ (NAC/ASA).

Results: Marked binge drinking (1.75 g ethanol/kg in 60 min) and blood alcohol levels exceeding 80 mg/dl were observed in the control group upon ethanol-re-access. *Lactobacillus*-GG or (NAC+ASA) treatments inhibited alcohol intake by 66–80%. The combination of both treatments virtually suppressed (inhibition of 90%) the re-access binge-like drinking, showing additive effects. Treatment with NAC+ASA increased the levels of glutamate transporters xCT and GLT-1 in nucleus accumbens, while Lactobacillus-GG administration increased those of the dopamine transporter (DAT).

Conclusions: The administration of a well-accepted probiotic may be of value as an adjunct in the treatment of alcohol-use-disorders.

1. Introduction

Studies in the past decade led to significant research demonstrating that gut microbiota composition has relevant effects on several brain neurotransmitter receptors and behaviors (Bravo et al., 2011; Hall et al., 2017; Leclercq et al., 2020). The influence of gut microbiota on ethanol intake was shown in subsequent studies (Zhao et al., 2020), demonstrating that transplantation of fecal material from alcoholic patients to mice, whose microbiota had been sharply suppressed by oral antibiotic administration, increased the preference of the animals for alcohol solutions. Subsequent studies showed that in rats of a line selectively bred as alcohol consumers, the oral treatment with non-absorbable

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antibiotics before ethanol exposure markedly reduced their ethanol intake (Ezquer et al., 2021). These studies also showed that the daily oral administration of *Lactobacillus rhamnosus-GG* (LGG) inhibited alcohol intake. Such an effect was consistent with the markedly increased levels of systemic fibroblast-growth factor 21 (FGF21) induced by *LGG* administration (Ezquer et al., 2021), which via the dopaminergic system has been reported to inhibit ethanol intake both in rodents and in humans (Talukdar et al., 2016). While in the latter studies, the effects of gut microbiota and of LGG on alcohol intake were assessed in animals that had not consumed ethanol prior to the antibiotic or Lactobacilli administration, the present study is first in assessing the effect of LGG administration on alcohol relapse.

Previous studies (Israel et al., 2021; Quintanilla et al., 2020) have shown that the relapse ethanol intake that follows ethanol re-access in chronically alcohol-treated and deprived animals is markedly inhibited by the oral administration of N-acetylcysteine and acetylsalicylic acid (NAC+ASA). Such and inhibitory effect of NAC+ASA on alcohol drinking relapse was associated with changes that tone-down the activated glutamatergic system in nucleus accumbens, as seen by increases in the glutamate transporters GLT-1 and xCT. NAC+ASA treatment also showed a marked blockade of alcohol-induced hippocampal oxidative stress.

Thus, it is hypothesized that LGG administration following oral antibiotic treatment will show additive effects on ethanol relapse to those of NAC+ASA. Such a hypothesis was tested in animals that after having ingested ethanol chronically were alcohol-deprived and received (i) LGG; (ii) NAC+ASA; (III) (LGG + NAC-ASA) or (iv) vehicle prior to being exposed to the relapse drinking condition induced by the re-access to alcohol, a condition leading to an marked increased ethanol intake referred to as the "alcohol deprivation effect" (ADE) (Karahanian et al., 2015; Rodd-Henricks et al., 2001; Spanagel and Holter, 1999; Tampier et al., 2013).

Thus, the effect of microbiota and LGG on ethanol relapse, either by themselves or combined with N-acetylcysteine + acetylsalicylic acid (NAC+ASA) treatment was investigated.

2. Materials and methods

2.1. Animals

Two-month-old male Wistar-derived rats, selectively bred for over 90 generations as ethanol consumers (University of Chile Bibulous; UChB) (Israel et al., 2017; Quintanilla et al., 2006) were used in the experiments. Animals were housed individually in acrylic cages and maintained on a 12–hour light/dark cycle (lights off at 7:00 PM) and regularly fed a soy protein, peanut-meal rodent diet (Cisternas, Santiago, Chile). Bedding consisted of corn husk. Experimental procedures were approved by the Ethics Committee for Experiments with Laboratory Animals at the Medical Faculty of the University of Chile (Protocol CBA# 0994 FMUCH) and the Chilean National Research and Development Agency (ANID).

2.2. Drugs

Ethanol solutions were prepared from absolute (99.99%) ethanol (Merck, Darmstadt Germany) diluted to 10% or 20% (v/v) in tap water. The combination of Neomycin (Cat Nº 1876) (Sigma-Aldrich, St. Louis, MO) plus polymyxin-B (Cat Nº 4932) (Sigma-Aldrich, St. Louis, MO) was dissolved in distilled water, and administered by the oral gavage in a volume of 6 ml/kg/day for seven days. The combination of N-acetylcysteine (NAC) (Sigma-Aldrich, St. Louis, MO) plus acetylsalicylic acid (ASA) (Sigma-Aldrich, St. Louis, MO) was dissolved in distilled water, adjusted with NaOH to pH 7.2, and administered by the oral gavage in a volume of 6 ml/kg/day for seven days. 5×10^9 CFU of *Lactobacillus rhamnosus* Gorbach-Goldin (LGG) (Vivera ®, Probiotica S.p. A; Novara, Italy) were resuspended in 1 ml of phosphate-buffered saline (PBS), pH 7.2 or vehicle (1 ml of PBS solution) and administered by the oral gavage for seven days.

2.3. Experiment 1.-effect of the intragastric administration of antibiotics on alcohol relapse and gut microbiota composition

Alcohol relapse was assessed by the alcohol deprivation effect (ADE). The ADE refers to the increased ethanol intake seen in animals that have chronically consumed ethanol, are thereafter alcohol-deprived for a long period and are subsequently allowed re-access to ethanol (Rodd-Henricks et al., 2001; Spanagel and Holter, 1999; Tampier et al., 2013). Twelve male naïve UChB rats, weighing 190–210 g, were single housed in cages at the age of 60 days and maintained on a 12-hour light/dark cycle (lights off at 7:00 PM). Subsequently, rats received continuous free-choice access to 10% (v/v) ethanol solution and water concurrently for 33 consecutive days. On day 33, after measuring ethanol consumption, fresh stool samples were collected and stored at − 80 °C until DNA analysis of the intestinal microbiome. On day 34 the animals were deprived of ethanol for 12 consecutive days (day 34–45). Water was continuously available during all the experiment. After a six-day period of deprivation, rats were divided into two groups (n = 6 per group) that on the last seven days of ethanol deprivation (day 39 to day 45) received one of the following daily treatments: (1) **Vehicle group**: rats were administered distilled water (6 ml/kg/day), by oral gavage; (2) **Antibiotics group**: rats were administered a combination of two non-absorbable antibiotics neomycin (250 mg/kg/day) and polymyxin-B (9 mg/kg/day) in volume of 6 ml/kg/day by oral gavage as previously reported (Davey et al., 2013; Ezquer et al., 2021). On the last day of alcohol deprivation and antibiotics/vehicle administration (day 45), fresh stool samples of both the vehicle and antibiotic-treated animals were collected and immediately stored at − 80 °C until the analysis of the gut microbiome DNA. On day 46, all rats were allowed 10% ethanol re-access and the amount of alcohol consumed was recorded and expressed as g of ethanol consumed/kg body weight. Animals were euthanized after completing the ethanol intake.

2.4. Blood ethanol determination

Blood ethanol levels were determined in animals that had consumed alcohol chronically, were alcohol deprived for 12 days, and were offered ethanol re-access. Immediately, after 60 min of re-access to ethanol (water also available), 100 µl of blood were collected from the tip of the tail under moderate acepromazine sedation (1 mg/kg i.p.). Samples were immediately mixed with 1.2 ml of saline at 4 °C, centrifuged to discard cells and the supernatant was analyzed by enzymatic analysis. Ten microliters of sample or standards were incubated in 1 ml buffer containing 0.1 M Tris-HCl pH 9.0, 1 mM NAD^+,10 mM semicarbazide and yeast alcohol dehydrogenase (Sigma-Aldrich, St Louis MO) at 37ºC. The absorbance of NADH was determined at 340 nm after the alcohol dehydrogenase reaction proceeded to completion.

2.5. Gut microbiome analysis

DNA from the fecal samples indicated above 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 16 S rRNA V4 region, and 2 × 250 bp paired-end sequencing was done using an Illumina HiSeq 2500 in rapid run mode. Raw sequencing data was curated and analyzed using High-Performance Computing (HPC) power resources at the UDD-Bioinformatics Center using the DADA2 pipeline (Callahan et al., 2016). In summary, all sequences were filtered to truncate the paired reads to 150nt 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 (Pruesse et al., 2007).

The resulting amplicon sequence variants (ASVs) were analyzed using the Phyloseq package in R, and chimeric sequences were removed using the Chimera Slayer algorithm (Haas et al., 2011). 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.

2.6. Experiment 2.- effect of the co-administration of antibiotics and Lactobacillus and of N-acetylcysteine (NAC) plus acetylsalicylic acid (ASA) on alcohol relapse

A new group of twenty-four male naïve UChB rats, weighing 190–210 g, were single housed in cages at the age of 60 days and as above maintained on a 12-hour light/dark cycle (lights off at 7:00 PM). Subsequently, rats received continuous concurrent free-choice access in their home cage to 10% (v/v) ethanol solution and water for 17 consecutive days. On day 18 rats were allowed three-bottle choice concurrent access to 10% and 20% (v/v) ethanol solutions and water for 16 additional days. The addition of a second more concentrated ethanol solution could allow a greater ethanol intake in a short (60 min) time. From day 34 to day 47, animals were deprived of ethanol for 14 consecutive days. On the first deprivation day rats were divided into four groups (n = 6 per group), namely: **(1) Vehicle/vehicle group**: rats were administered distilled water (6 ml/kg/day), by oral gavage, for the fourteen deprivation days; **(2) Antibiotics/*Lactobacillus* group**: on the first seven days of deprivation (from day 34–40) rats were administered a combination of two antibiotics neomycin (250 mg/kg/day) and polymyxin B (9 mg/kg/day) in a volume of 6 ml/kg/day by oral gavage daily as previously reported (Davey et al., 2013; Ezquer et al., 2021), and subsequently, during the last seven days of deprivation (from day 41–47), rats were administered a daily suspension of 5×10^9 CFU *Lactobacillus rhamnosus* Gorbach-Goldin (LGG) (Vivera ®, Probiotica S.p. A; Novara, Italy) resuspended in 1 ml of phosphate-buffered saline (PBS), **(3) Vehicle/NAC+ASA group**: on the first seven days of deprivation (from day 34–40) rats were administered distilled water (6 ml/kg/day), by oral gavage, and subsequently, during the last seven days of deprivation (from day 41–47), they were daily administered N-acetylcysteine (40 mg/kg) + acetylsalicylic acid (15 mg/kg) (6 ml/kg/day) by oral gavage. The doses of NAC and ASA were selected following (Israel et al., 2021) (Quintanilla et al., 2020) **(4) Antibiotics/*Lactobacillus*/NAC+ASA group**: On the first seven days of deprivation (from day 34–40) rats were administered the a combination of the two antibiotics neomycin (250 mg/kg/day) and polymyxin B (9 mg/kg/day) in a volume of 6 ml/kg/day, by oral gavage, and subsequently, during the last seven days of deprivation (from day 41–47), they were administered first a daily suspension of 5×10^9 CFU *Lactobacillus rhamnosus* Gorbach-Goldin (LGG) (Vivera ®, Probiotica S.p.A; Novara, Italy) resuspended in 1 ml of phosphate-buffered saline (PBS) by oral gavage, and after twenty minutes animals were administered the combination of N-acetylcysteine (40 mg/kg) + acetylsalicylic acid (15 mg/kg) (6 ml/kg/day) by oral gavage. Thereafter, on day 48, all rats were allowed re-access to the ethanol solutions (10% and 20% v/v concurrently) and water, for three days (day 48–51) and the first hour (60-min) and daily (24-hour) ethanol intakes were recorded and expressed as g of ethanol consumed/kg body weight. On day 52, all animals were subjected to a second additional cycle of seven days of ethanol deprivation (day 52–58) before another three days of ethanol re-access. An additional deprivation cycle has been reported to enhance the ethanol-deprivation effect (Karahanian et al., 2015; Rodd-Henricks et al., 2001; Tampier et al., 2013). During this second 7-day deprivation period, animals of each group received the same drug combination or vehicle treatment as received during the first ethanol deprivation period.

Thereafter, all rats were allowed re-access to the ethanol solutions (10% and 20% v/v) and water, for three days (days 59 through 62) and both first hour (60-minute) and daily ethanol (24-hours) intakes were recorded and expressed as g of ethanol consumed/kg body weight. Immediately after completing and recording the alcohol intakes on the third 24-hour deprivation-re-access cycle, animals were anaesthetized by intramuscular administration of ketamine (90 mg/kg)/xylazine (10 mg/kg) to obtain blood samples by cardiac puncture and perfused intracardially with 100 ml of 0.1 M PBS (pH 7.4) to obtain brain samples.

2.7. Determination of GSSG/GSH ratio in hippocampus

Hippocampus were extracted and mixed with three volumes of ice-cold potassium buffer containing 5 mM EDTA, pH 7.4, flash-frozen and stored at − 80 °C until homogenization. Reduced glutathione (GSH) and glutathione disulfide (GSSG) contents were determined as described previously (Perez-Lobos et al., 2017; Rahman et al., 2006). Briefly, GSSG in the sample was first converted into GSH with glutathione reductase and NADPH. The total free thiol group of GSH was reacted with the sulfhydryl reagent DTNB (5,50-dithiobis-2- nitrobenzoic acid) yielding a product that absorbs at 412 nm. GSSG per se in the homogenate was measured by adding 2-vinyl pyridine, a thiol scavenger that traps GSH, preventing GSH from binding to DNTB. The excess of 2-vinyl pyridine was neutralized with triethanolamine. Thereafter, GSSG was converted into GSH by glutathione reductase and NADPH, DNTB was added, and absorbance measured at 412 nm.

2.8. Determination of xCT and GLT-1 glutamate transporters and dopamine transporter (DAT) levels in nucleus accumbens

Total proteins in nucleus accumbens samples were extracted using T-per lysis buffer (Thermo-Fisher) containing protease inhibitors. Western blot procedures were performed using 25 μg of proteins to examine the level of GLT-1 detected with a guinea pig anti-GLT-1 polyclonal primary antibody (Cat AB1783, Millipore, 1:500 dilution) and a IRDye 800CW donkey anti-guinea pig secondary antibody (Cat 925–32411, LI-COR, 1:10000 dilution); xCT detected with a rabbit anti-xCT monoclonal primary antibody (Cat AB175186, Abcam, 1:500 dilution) and a IRDye 800CW donkey anti-rabbit secondary antibody (Cat 926–32213, LI-COR, 1:10 000 dilution) and DAT detected with a rabbit anti-DAT monoclonal primary antibody (Cat AB184451, Abcam, 1:500 dilution) and a IRDye 800CW donkey anti-rabbit secondary antibody (Cat 926–32213, LI-COR, 1:10 000 dilution). The same membranes were assessed for β-actin immunoblotting as loading control (mouse anti β-actin primary antibody, Cat sc-47778 Santa Cruz Biotechnology, 1:200 dilution and IRDye 800CW goat anti-mouse secondary antibody, Cat 926–32210, LI-COR). Reactive bands were detected using the Odyssey Imaging System (LI-COR) and quantified using the Image Studio Lite 5.2 software.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 8.0.2 (Jan 2019) (San Diego, CA). Data are expressed as means ± SEM. The normal distribution of data was confirmed for all experiments using the Shapiro-Wilk test. Bartlett's test was used to determine if the variances between various groups were equal. A level of $p > 0.05$ was considered indicative of homogeneity of variance. For normally distributed data with homogeneous variances as shown in this study, the one-way or two-way analysis of variance (ANOVA) was used followed by a Tukey post hoc test. When only two groups were compared, statistical significance was determined by Student's t-test. A level of $P < 0.05$ was considered for statistical significance. To facilitate text reading, full statistical ANOVA analyses are presented in Figure legends.

3. Results

Experiment 1 determined whether relapse alcohol intake is influenced by gut microbiota composition. Antibiotics were administered during an imposed alcohol deprivation period which followed 33 days of ethanol intake. The relative abundance of fecal microbiota species was analyzed on the last day of the alcohol deprivation, after which ethanol re-access was allowed and the amount of ethanol consumed was determined.

Fig. 1A right, shows that rats of the control group deprived of alcohol for 12 days following a period of 33 days of free access to 10% v/v ethanol solution and water consumed intoxicating levels of ethanol in the first 60 min of re-access to 10% ethanol; consumption that was inhibited by 35–40% ($p < 0.005$) by the combined oral administration of two non-absorbable antibiotics (neomycin; 250 mg/kg/day plus polymyxin-B; 9 mg/kg/day) given during the last 7 days of alcohol deprivation, immediately prior to ethanol re-access. Fig. 1B shows that the blood alcohol level after the 60–minute ethanol re-access was 129.0 ± 12 mg/dl in vehicle-treated animals (binge drinking in humans considered greater than 80 mg/dl). Blood ethanol levels of animals treated with neomycin plus polymyxin B was 60% lower than those of the vehicle group ($p < 0.001$), thus again indicating that relapse drinking was inhibited by antibiotic treatment. Fig. 1C, right shows that the extended 24-hour ethanol intake displayed by control animals (vehicle) on the first day of ethanol re-access was increased by 30% ($p < 0.0001$) over their baseline ethanol intake before deprivation. The higher ethanol intake of the control group on the first 24-hours of re-access is in line with the existence of an ADE. Neomycin plus polymyxin B treatment inhibited the first day re-access ethanol intake by 20% ($p < 0.02$) versus vehicle controls.

Fig. 2 shows the changes in microbiota profile determined by 16 S rRNA sequencing of microbiota DNA in feces of (i) naive animals; (ii) animals that ingested ethanol for 33 days; (iii) animals that had ingested ethanol for 33 days and were subjected to a 12-days deprivation period which included 7 days of the daily administration of vehicle or (iv) animals that had ingested ethanol for 33 days and were subjected to a 12-days deprivation period which included 7 days of the daily administration of non-absorbable antibiotics (ABXs: neomycin 250 mg/kg/day and polymyxin-B 9 mg/kg/day). As previously reported for ethanol-naïve animals (Ezquer et al., 2021), antibiotic administration to chronically ethanol consuming animals led to a reduction in the Chao-1 and Shannon α-diversity indices, whereas changes due to ethanol intake alone were much less pronounced (Fig. 2 A).

Subsequently, the composition of the gut microbiota at the phylum, family, and genus levels was analyzed (Fig. 2B-E). Noteworthy, antibiotic administration greatly reduced ($p < 0.01$) the relative abundance of Proteobacteria, a highly pro-inflammatory phylum previously associated with high alcohol intake (Kosnicki et al., 2019) and increases ($p < 0.001$) the relative abundance of phylum Bacteroidetes (Fig. 2C); a reduction in this phylum has been previously associated with high alcohol intake (Kosnicki et al., 2019). Additionally, a major difference at the genus levels was observed; a 4-fold increase in *Lactobacillus johnsonii* in antibiotic-treated animals (Fig. 2F), which might be an important factor leading or contributing to the observed reduction in ethanol intake. This is in line with our previous report (Ezquer et al., 2021) that in naive rats Lactobacillus rhamnosus (LGG) administration inhibited ethanol intake. Thus, as indicated above, antibiotic inhibition on relapse ethanol intake might be amplified by the exogenous administration of oral *Lactobacilli* to antibiotic treated animals. While *Lactobacillus johnsonii* is not commercially available as a pharmaceutical preparation, *Lactobacillus rhamnosus-GG (LGG)* is available and approved for human use (Capurso, 2019).

The effect on alcohol relapse of Antibiotic-LGG and its combination with N-acetylcysteine + acetylsalicylic acid (NAC+ASA) was addressed in Experiment 2 where the post-deprivation alcohol relapse intake was determined. The effect on the ethanol ADE-relapse intake (60-minute and 24-hour ethanol intakes) was assessed following the administration of (i) Antibiotic/*Lactobacillus rhamnosus*-GG, (ii) N-acetylcysteine plus acetylsalicylic acid (NAC+ASA); (iii) the combination of both above treatments and (iv) no treatments (vehicle/vehicle).

Fig. 3 shows that the initial 60-minute binge-like drinking (1.65–1.75 g ethanol/kg) upon ethanol re-access was inhibited by 66–80% ($p < 0.0001$) by either *Lactobacillus*-GG or (NAC+ASA) treatments. The combination of both treatments virtually suppressed (inhibition of 90%) the re-access binge-like drinking, showing additive effects ($p < 0.05$) on the initial re-access. Water-only intake in the first 60-minutes of ethanol re-access was negligible.

Fig. 4A shows that the control group (vehicle/vehicle) displayed an

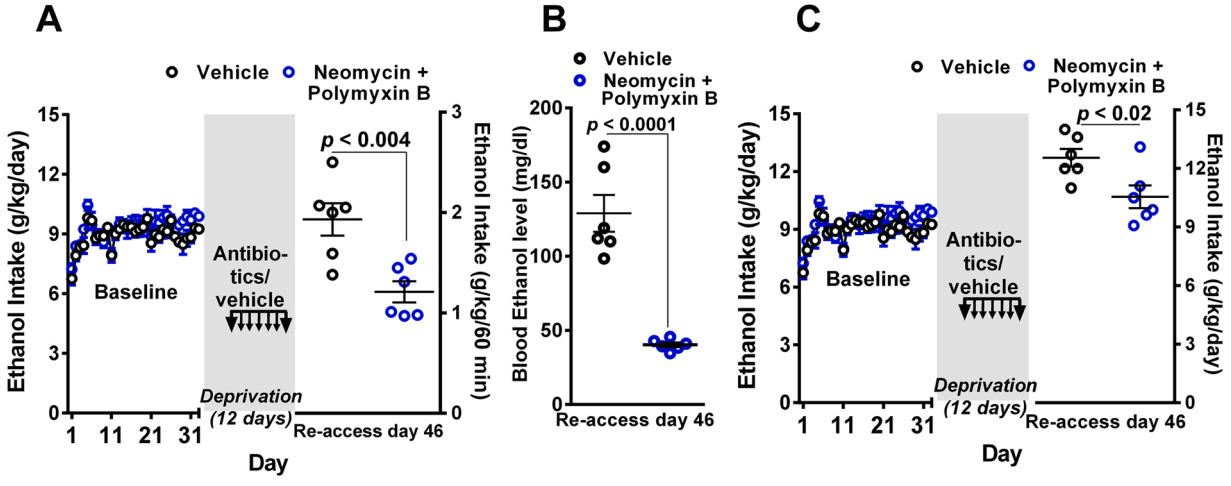

Fig. 1. Oral antibiotic administration during alcohol deprivation to rats that had previously ingested ethanol for 33 days reduced relapse binge-like drinking upon ethanol re-access. (A) The initial (60-minute) post-deprivation ethanol intake was inhibited by a 7-day oral administration of non-absorbable antibiotics (Neomycim + Polymyxin B) prior to ethanol re-access compared with the control group treated with water (** $p < 0.01$, Student t test). (B) The blood ethanol levels displayed by rats treated with neomycin + polymycin B after the 60-min ethanol intake were lower than those exhibited by control animals (****$p < 0.0001$ Student *t* test). (C) The daily ethanol intake displayed by the control group on the first day (day 46) of ethanol re-access was higher than the intake of the same group prior to the ethanol deprivation (baseline black empty circles), indicating the presence of an ADE (****$p < 0.0001$ first day of re-access of the vehicle group, compared with the mean intake of the last 10 days before deprivation of the same group, student *t* test). Antibiotic treatment marginally (15–20%) inhibited the 24-hour (ADE) ethanol intake compared to control animals (*$p < 0.02$ Student *t* test). Conversely, antibiotic treatment increased water intake ($p < 0.02$); $n = 6$ for each experimental condition.

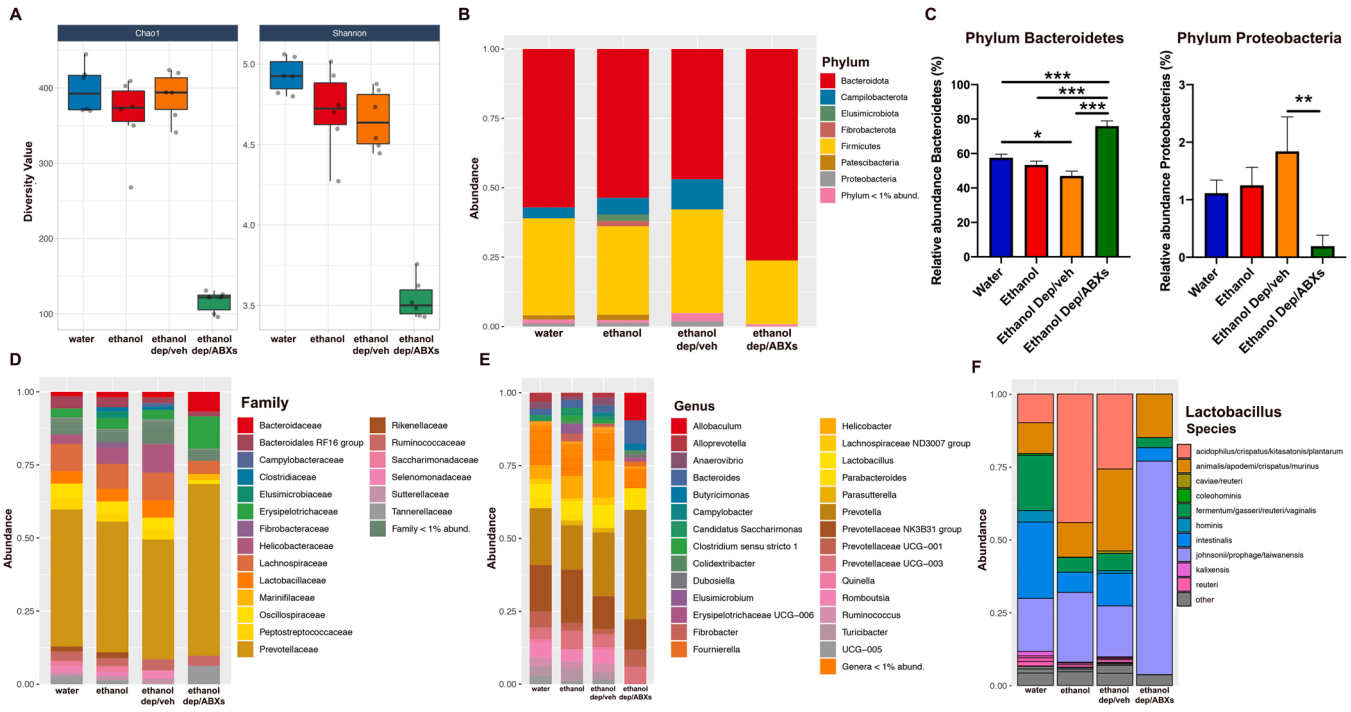

Fig. 2. Oral antibiotic administration during an alcohol deprivation period imposed following 33 days of ethanol consumption altered the composition of the gut microbiota. (A) Chao-1 and Shannon diversity indices (α-diversity) of ethanol consuming animals subjected to a 12-days deprivation period that were treated with daily oral administration of the non-absorbable antibiotics (Neomycim + Polymyxin B; ABXs) or vehicle during the last 7 days of the deprivation period. (B) Relative abundance at the phylum level. (C) Comparison of relative abundance of phyla Bacteroidetes and Proteobacteria. The antibiotic treatment increased the abundance of Bacteroidetes (*** $p < 0.001$, One-way ANOVA followed by Tukey's post-hoc test) while markedly reducing that of Proteobacteria (** $p < 0.01$), One-way ANOVA followed by Tukey's post-hoc test). (D) Relative abundance at the family level. (E) Relative abundance at the genus level. (F) Relative abundance of species of Lactobacillus of animals treated with antibiotics or vehicle for 7 days (n = 6 per group). A 4-fold increase in *Lactobacillus johnsonii* is seen. n = 6 for each experimental condition.

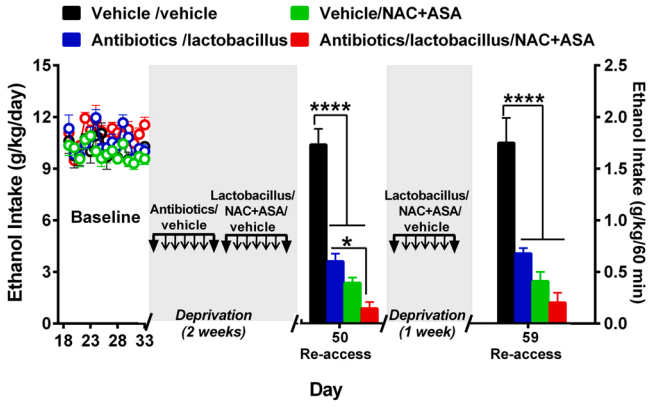

Fig. 3. Oral antibiotic administration followed by the administration of *Lactobacillus rhamnosus-GG and* of N-acetylcysteine + acetylsalicylic acid during alcohol deprivation to rats that had ingested ethanol for 33 days markedly reduced the 60-minute relapse binge-like drinking upon ethanol re-access. Additive treatment effects. Two-way ANOVA (treatment × day) of the 60-min ethanol intake after the first and second re-access cycles revealed a clear effect of treatment ($F_{treatment(3,38)} = 38.55$ * ** * $p < 0.0001$), but not of day, while there was no significant treatment × day interaction. Tukey's post hoc analysis indicated that, compared with the ethanol intake of the control group (vehicle/vehicle), the ethanol intakes of the antibiotics/lactobacillus group, the vehicle/NAC+ASA group and antibiotics/lactobacillus/NAC+ASA group were lower during the first and second cycle of ethanol re-access. Tukey's post hoc analysis indicated that the co-administration of antibiotics/lactobacillus plus NAC+ASA induced an inhibition of ethanol intake that was stronger compared to that of the group treated with antibiotics/lactobacillus, indicating an additive effect of treatments following the first deprivation (*$p < 0.05$). All data are presented as mean ± SEM; n = 6 for each experimental condition.

increased ($p < 0.01$) ethanol intake (ADE) on the first 24-hours of alcohol re-access versus pre-deprivation baseline ethanol intake; increase which was enhanced ($p < 0.01$) on the second ethanol-re-access cycle. The oral administration of either Antibiotic/*Lactobacillus rhamnosus*-GG or oral administration of N-acetylcysteine + Acetylsalicylic acid inhibited the 24-hour ethanol relapse intake by 66–70% ($p < 0.0001$), while the inhibitory effect of the combination of both treatments showed additive effects ($p < 0.05$), reaching reductions of ethanol intake of 75–85% ($p < 0.05$). **(B)** Figure shows that the inhibition of ethanol intake induced by Antibiotic/*Lactobacilli* or NAC+ASA treatments was in all cases replaced by water intake. Water intakes in Fig. 4B are essentially a mirror image of the effects of ethanol intakes shown in Fig. 4A, indicating that the reduction in the intake of ethanol solutions by the different treatments was replaced by an increase of water intake.

Supplementary Fig. 1 displays total daily fluid intake and animals body weights, showing that these parameters were not affected by the *Lactobacilli* or NAC+ASA treatments, thus indicating that the therapeutic effects induced by these treatments, or their combination were specific for alcohol intake.

Subsequent studies investigated the possible mechanisms by which *Lactobacilli* and NAC+ASA treatments might act in reducing alcohol relapse. We previously reported (Israel et al., 2021; Quintanilla et al., 2020) that rats that engage in a marked post deprivation binge-like drinking display hippocampal brain oxidative stress, as shown by elevations in the ratio of oxidized glutathione/reduced glutathione (GSSG/GSH). Fig. 5 shows that both NAC+ASA and *Lactobacilli* treatments reduced the oxidative stress induced in hippocampus by 33 days of continuous ethanol intake. The inhibitory effect of Lactobacilli treatment on the GSSG/GSH ratio was however less remarkable than that of NAC+ASA treatment.

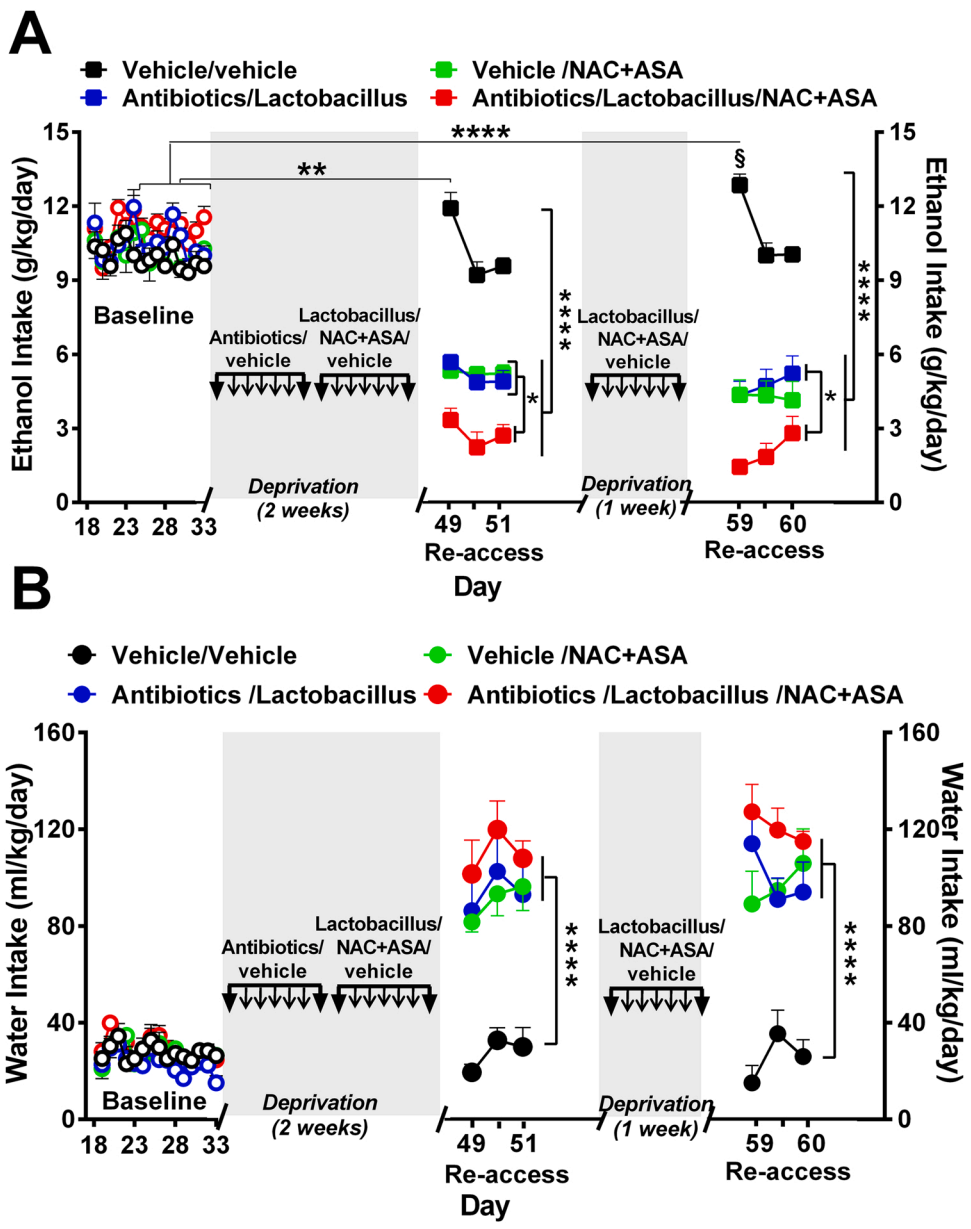

Fig. 4. Oral antibiotic administration followed by the administration of *Lactobacillus rhamnosus-GG and* of N-acetylcysteine + acetylsalicylic acid during alcohol deprivation to rats that had ingested ethanol for 33 days markedly reduced the 24-hour relapse intake upon ethanol re-access and increased water intake. Combined treatment effects. (A) The ethanol intake displayed by the control group (vehicle/vehicle) on the first day of both the first and second access cycles, was significantly higher, $p < 0.01$ and $p < 0.0001$, than the mean ± SEM ethanol intake displayed by rats of the control group on the last ten baseline days prior to alcohol deprivation, indicating the presence of an ADE. In addition, the control group showed higher ethanol intake on the first day of the second re-access cycle than on the first day of the first re-access cycle (§ $p < 0.01$), indicating that repeated deprivations increased ethanol intake, as shown in the first day of re-exposure. Two-way ANOVA (treatment × day) of the daily ethanol intake during the first and second re-access cycles revealed an effect of treatment [$F_{treatment(3114)} = 236.1$ * ** * $p < 0.0001$], day [$F_{day(5,114)} = 2.922$ * $p < 0.01$] and a treatment × day interaction [$F_{(15114)} = 2.175$, $p < 0.01$]. Tukey's post hoc analysis indicated that compared with the ethanol intake of the control group (Vehicle/vehicle), the ethanol intakes displayed by the antibiotics/lactobacillus group, the vehicle/NAC+ASA group and the antibiotics/lactobacillus/NAC+ASA groups were lower during the first and second cycles of ethanol re-access (*** $p < 0.0001$). *Post hoc* analysis indicated that the co-administration of antibiotics/lactobacillus plus NAC+ASA induced an inhibition of ethanol intake that was higher versus that of the group treated with antibiotics/lactobacillus and vehicle/NAC+ASA during the first re-access cycle and only antibiotics/lactobacillus during both re-access cycles (*$p < 0.05$), indicating additive treatment effects. (B) Water daily intakes of animals in the same conditions as in Fig. 4 A show that the reduction in the intake of ethanol solutions by the different treatments was replaced by an increase of water intake, maintaining animal's hydric balance. All data are presented as mean ± SEM; n = 6 for each experimental condition.

Addressing the likely mechanism of the additive effects of (NAC+ASA) and LGG on alcohol relapse, Fig. 6A and 6B show that NAC+ASA treatment but not *Lactobacilli* treatment increased the protein levels of xCT (Fig. 6A) and GLT-1 in nucleus accumbens (Fig. 6B), two important glutamate transporters known to reduce the alcohol-induced hyper-glutamatergic tone, which have been previously shown to be associated with a reduced ethanol relapse (Israel et al., 2021; Quintanilla et al., 2020; Sari, 2014). Additionally, consistent with previous studies (Ezquer et al., 2021; Talukdar et al., 2016), Fig. 6C shows that levels of the dopamine transporter (DAT), the main mechanism of dopamine removal from the synapse, were increased in the nucleus accumbens of animals that received either LGG or LGG+ NAC+ASA when compared with that of control rats (vehicle/vehicle) but not in animals that received only NAC+ASA (see Discussion).

4. Discussion

It is generally accepted for drugs of abuse, including alcohol, that the post-deprivation relapse involves a cue-increased glutamatergic tone at the nucleus accumbens tripartite synapse (Gass et al., 2011; Kalivas et al., 2009; Scofield et al., 2016). Two important mechanisms can reduce the glutamatergic tone; one is the increase in astrocyte glutamate transporter GLT-1, which acts by removing extracellular glutamate. Increased GLT-1 levels lead to an inhibition of chronic alcohol intake and post-deprivation alcohol relapse (Israel et al., 2021; Sari, 2014; Sari et al., 2016). A second mechanism that reduces a hyper-glutamatergic tone is the activation of the xCT (cysteine/glutamate) antiport activity by cystine (Baker et al., 2003). N-acetylcysteine administration, by increasing cystine levels, reduces chronic alcohol intake and alcohol relapse via the activation of xCT, which extrudes intracellular glutamate and activates the metabotropic (inhibitory) mGLU2/3 receptors in pre-synaptic neurons (Quintanilla et al., 2020).

Noteworthy, the tripartite post-synaptic medium spiny neurons of nucleus accumbens receive not only input from glutamate, but also from dopaminergic neurons (Scofield et al., 2016). Ethanol intake increases dopamine release in the nucleus accumbens (Bustamante et al., 2008; Di

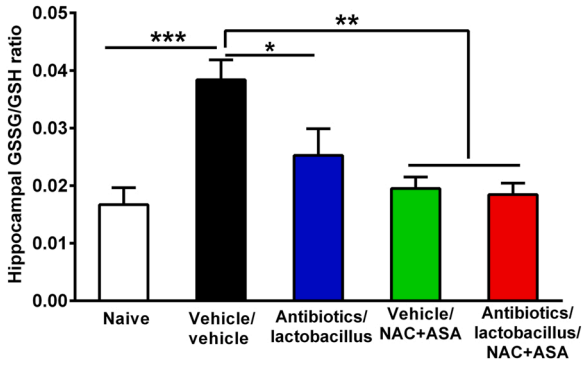

Fig. 5. The increased hippocampal oxidized glutathione/reduced glutathione (GSSG/GSH) ratio that followed 33 days of ethanol intake, 7 days of ethanol deprivation and ethanol re-access was inhibited by the administration of antibiotics + *Lactobacillus rhamnosus–GG or the administration of N-acetylcysteine + acetylsalicylic acid*. Oxidative stress determined as the ratio of oxidized/reduced glutathione (GSSG/GSH) is markedly increased (200%) by chronic ethanol intake/deprivation and re-access versus the water group (naive group [$F_{treatment(4,25)} = 7.852$ * ** $p < 0.001$; Tukey post hoc: water versus ethanol vehicle, * ** $p < 0.001$], whereas GSSG/GSH ratio in the Antibiotics/*Lactobacillus* group, vehicle/NAC+ASA group and Antibiotics/Lactobacillus/NAC+ASA group were fully normalized compared to the ethanol vehicle group (* $p < 0.05$; ** $p < 0.001$). Al data are presented as mean ± SEM; n = 6 for each experimental condition.

Chiara and Imperato, 1988; Karahanian et al., 2011). Thus, means to reduce dopaminergic activity are expected to reduce ethanol relapse. *Lactobacillus* administration leads to the hepatic generation of fibroblast growth factor 21 (FGF-21) (Zhao et al., 2019), which in *naive* mice inhibits alcohol intake by increasing dopamine transporter (DAT) (Talukdar et al., 2016), thus reducing free post-synaptic dopamine availability. An increased level of DAT following LGG administration was confirmed in the present study.

The present study is the first report showing that LGG supplementation inhibits alcohol relapse. From the above, data obtained point to the presence of two mechanisms induced by (NAC+ASA) and (antibiotic+LGG) which reduce the ADE effect independently and complementary, due to a reduced glutamatergic tone and a reduced dopaminergic tone respectively. An inhibitor of the xCT glutamate exchanger has already been shown to block the inhibitory effect of NAC on ADE (Quintanilla et al., 2020), and the levels of the cystine/glutamate transporter xCT were also shown to be increased by the combination of NAC+ASA (Israel et al., 2021). Future studies should interrogate the dopaminergic mechanism using specific inhibitors.

Oral antibiotic administration during the deprivation period, prior to allowing ethanol re-access, led to a moderate inhibition (37% $p < 0.01$) of early (60-min) alcohol relapse. The inhibitory effect on relapse ethanol intake was markedly increased (66% $p < 0.0001$) when oral *Lactobacillus rhamnosus-GG* was administered after antibiotic treatment, prior to offering alcohol re-access in the alcohol deprivation (ADE) condition model.

As previously reported, the administration of N-acetylcysteine + Acetylsalicylic acid inhibited early (60-min) alcohol relapse by 70–75% (Israel et al., 2021). Further, alcohol relapse was virtually abolished (90% $p < 0.0001$) in animals that received *both Lactobacillus rhamnosus*-GG and N-acetylcysteine + acetylsalicylic acid treatments prior to allowing alcohol re-access. An additive inhibitory effect on ethanol relapse intake of both treatments was seen ($p < 0.05$ to $p < 0.01$) whether examined at the early (60-minute) ethanol intake or when relapse intake was determined following 24 h of alcohol re-access.

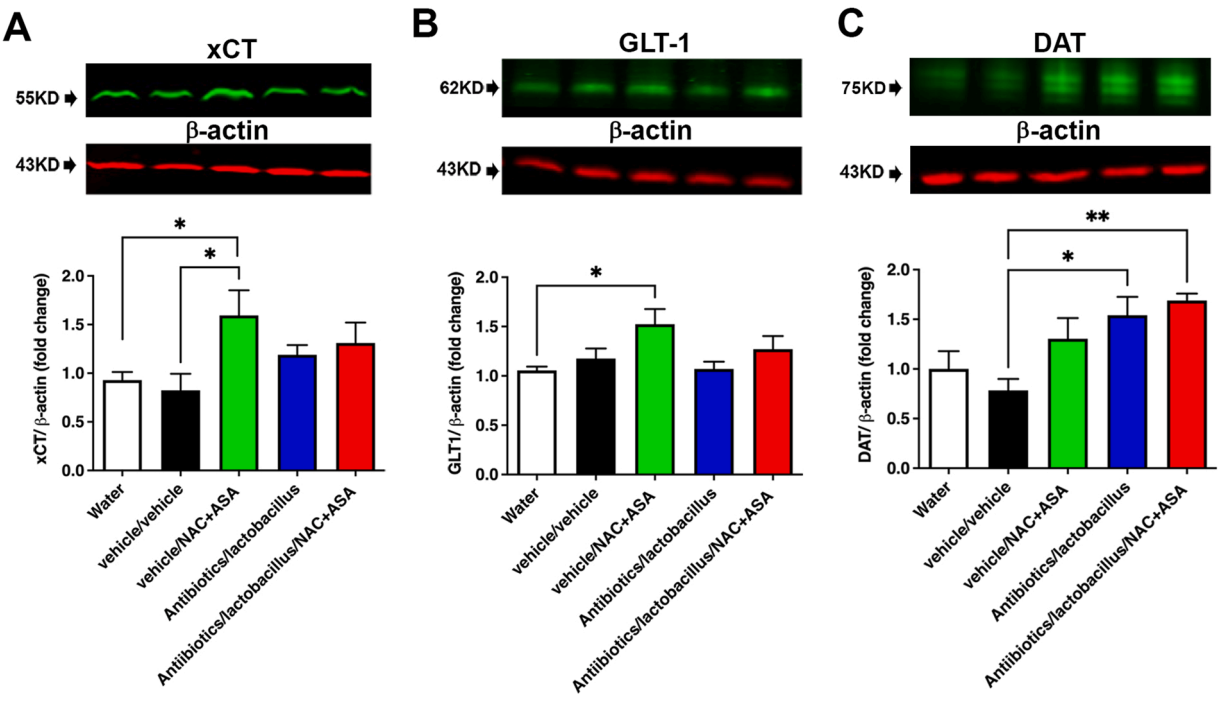

Fig. 6. Oral NAC+ASA administration during alcohol deprivation increased glutamate transporters levels while oral antibiotics + *Lactobacillus rhamnosus–GG* administration increased dopamine transporter level in nucleus accumbens. (A) xCT protein levels, (B) GLT-1 protein levels and (C) DAT protein levels in nucleus accumbens. xCT and GLT-1 levels of rats drinking ethanol chronically and treated with NAC+ASA during the last seven days of the first and second deprivation periods and allowed alcohol re-access were increased compared with their levels in animals chronically drinking ethanol (vehicle/vehicle group) or drinking only water (naive group) (One-way ANOVA followed by Tukey's post *hoc* * $p < 0.05$). DAT levels of rats drinking ethanol chronically and treated with Antibiotics/*Lactobacillus* or with Antibiotics/Lactobacillus/NAC+ASA during the last seven days of the first and second deprivation periods and allowed alcohol re-access were increased compared with their levels in animals chronically drinking ethanol (vehicle/vehicle group) (One-way ANOVA followed by Tukey's post *hoc,* * $p < 0.05$ for Antibiotics/*Lactobacillus* and ** $p < 0.01$ for Antibiotics/Lactobacillus/NAC+ASA*)*. Data are presented as xCT/β-actin, GLT-1/β-actin or DAT/β-actin ratio, relative to control levels. Data are shown as mean ± SEM. Immunoblots shown are representative of n = 6 per experimental condition.

While the present study strongly suggest that the mechanisms of LGG and NAC+ASA action are different (Fig. 6A, B and C), it was recently reported that N-acetylcysteine can increase the levels of gut *Lactobacilli* in a dysbiotic microbiota (Zheng et al., 2019), which may in part add to an alcohol anti-relapse effect.

It was previously reported that treatment with acetylsalicylic acid increases glutamate transporter GLT-1 levels (Israel et al., 2021). In the present study it was further shown that acetylsalicylic acid + N-acetylcysteine administration also increased the GLT-1 protein levels in nucleus accumbens. Additionally, data showed that the protein levels of the cystine/glutamate transporter xCT were also increased by the combination of acetylsalicylic acid + N-acetylcysteine. Such a combination is of translational value as it mimics the effects of ceftriaxone (Rao et al., 2015), which being a beta-lactam antibiotic could not be used chronically as an adjuvant in the treatment of alcoholism.

Unexpectedly, the increases in xCT or GLT-1 levels induced by acetylsalicylic acid + N-acetylcysteine were not seen in a condition in which the latter were combined with Antibiotic/*Lactobacilli* administration. While the reason for this observation is not clear, the effects of N-acetylcysteine in increasing the xCT-mediated glutamate exchange per se via cystine as a substrate, and mGlu2/3 receptor activation (Quintanilla et al., 2020) could add to the DAT-increased dopaminergic inhibition induced by *Lactobacilli* administration (Talukdar et al., 2016), as confirmed in the present study.

As reported earlier, chronic ethanol treatment leads to marked hippocampal oxidative stress (Fernandez-Rodriguez et al., 2022; Israel et al., 2021; Johnsen-Soriano et al., 2007; Quintanilla et al., 2020). Present studies confirm the generation of hippocampal oxidative stress, assessed by increases in the GSSG/GSH ratio in animals that had consumed ethanol for one month, were deprived for 14 days and were allowed re-access to the ethanol solutions. It is of interest that Antibiotic/Lactobacillus treatment also led to a reduction of oxidative stress, although to a lesser degree than the full inhibition of oxidative stress exerted by NAC+ASA. Considering that the activity of the glutamate transporter GLT-1 is dependent on oxidative stress (Trotti et al., 1998; Volterra et al., 1994), the possibility that part of the antibiotic/*Lactobacilli* anti-relapse effect per se might be exerted via an increased GLT-1 activity -rather than on the amount of the transporter- cannot be discarded.

It is noted that in the present studies, rats that consumed 10 g ethanol/kg/day for one month, showed a greatly elevated GSSG/GSH ratio even though the animals were deprived for 14 days and further allowed alcohol re-access, which was available until animal euthanasia. An elevated GSSG/GSH ratio in the hippocampus of rats after alcohol re-access has been a constant finding in the present high alcohol intake rat model (Ezquer et al., 2019; Israel et al., 2021; Quintanilla et al., 2020, 2019). In a recent study in rats (Fernandez-Rodriguez et al., 2022), the chronic ethanol intake of the order of 2–2.5 g ethanol/kg/day led to increases in the hippocampal GSSG/GSH ratio upon ethanol withdrawal, while such ratio returned to normal levels upon ethanol re-access. The reason for the difference between these studies is not certain but may relate to differences in alcohol volition in the animals of these studies.

4.1. Limitations of the study

It is noted that multiple doses of the inhibitors were not tested, and animal food intake was not determined. Additionally, female rats were not included in the study. It is also noted that the reductions in total ethanol intake reported in the study do not distinguish between changes in early ethanol intake (likely odor-cued craving) and final satiety. Similarly, future studies are needed to determine the possible role of *Lactobacillus rhamnosus*-GG on ethanol absorption and on its metabolism into acetaldehyde.

5. Conclusions

The present study indicates that the administration of drugs that inhibit the hyper-glutamatergic state (N-acetylcysteine and acetylsalicylic acid) along with the administration of a probiotic (*Lactobacillus rhamnosus)* reported to inhibit the dopaminergic tone, markedly inhibit relapse ethanol intake. Translationally, the oral administration of a clinically well-accepted probiotic, such as the *Lactobacillus rhamnosus-GG*, may be of value as an adjunct in interventions addressing alcohol use disorders.

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CRediT authorship contribution statement

FE, MEQ, YI designed the study, JMM and FM-F analyzed the bacterial DNA, PM, DS ME and MHM contributed to data collection and data presentation. All authors approved the manuscript.

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Conflicts of interest

Authors declare no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.drugalcdep.2022.109466.

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