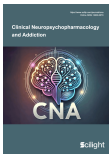
Scilight

Clinical Neuropsychopharmacology and Addiction

https://www.sciltp.com/journals/cna



Article

Check for updates

The Role of Probiotic Supplementation in Alcohol-Seeking Behavior: Behavioral and Neurochemical Correlates

Huma Ikram [1,*], Shahla Perveen [1] and Darakhshan J. Haleem [1,2]

[1] Neurochemistry and Biochemical Neuropharmacology Research Unit, Department of Biochemistry, University of Karachi, Karachi 75270, Pakistan

[2] Neuroscience Research Laboratory, Dr. Panjwani Center for Molecular Medicine and Drug Research, University of Karachi, Karachi 75270, Pakistan

* Correspondence: huma_biochemist@yahoo.com

How To Cite: Ikram, H.; Perveen, S.; Haleem, D.J. The Role of Probiotic Supplementation in Alcohol-Seeking Behavior: Behavioral and Neurochemical Correlates. *Clinical Neuropsychopharmacology and Addiction* **2026**, *2*(2), 8. https://doi.org/10.53941/cna.2026.100008

Received: 21 December 2025
Revised: 6 April 2026
Accepted: 7 May 2026
Published: 1 June 2026

Abstract: Background: Alcohol Use Disorder (AUD) is depicted by persistent craving and repeated administration, underpinned by neuroinflammation, oxidative stress, and dysregulation of reward pathways. Emerging evidence suggests a role for gut-brain communication of these processes, with gut dysbiosis exacerbating behavioral and neurochemical correlates of addiction. Purpose: This investigation aimed to evaluate the therapeutic efficacy of targeted probiotic supplementation in modulating alcohol-seeking behavior and its underlying neurobiological substrates. Study design: Preclinical controlled study. Methods: Using a pre-clinical rodent model of AUD, subjects were administered a specific multi-strain probiotic formulation alongside chronic intermittent ethanol exposure. Behavioral assays were employed to quantify alcohol preference and reward-motivated behaviour (using conditioned place preference). Parallel biochemical analyses assessed systemic and central anti-inflammatory markers and antioxidant capacity. Levels of serotonin, dopamine and metabolites were estimation in caudate and nucleus accumbens via high performance liquid chromatography. Results: Probiotic administration significantly reduced alcohol preference and attenuated conditioned place preference for ethanol-paired contexts compared to controls. These behavioral improvements correlated with robust anti-inflammatory and antioxidant effects, as evidenced by reduced pro-inflammatory cytokines and enhanced oxidative stress defense in both plasma and brain tissue. Critically, probiotic treatment normalized neurochemical dysregulation, increasing tonic GABAergic inhibition and restoring dopamine dynamics within the reward circuitry. Conclusion: These findings demonstrate that probiotic supplementation effectively reduces alcohol seeking behavior and reward-related behaviors by mitigating neuroinflammation, oxidative stress, and aberrant neurotransmission. The results highlight the gut-brain axis as a viable target for novel nutritional interventions in AUD and provide a mechanistic rationale for the adjunct use of probiotics in managing alcohol dependence.

Keywords: probiotic; alcohol use disorder; gut-brain axis; conditioned place preference; neuroinflammation; oxidative stress; dopamine

1. Introduction

The compulsive consumption of alcohol, clinically recognized as Alcohol Use Disorder (AUD), imposes a severe and persistent burden on global public health systems [1]. This chronic, relapsing condition is marked by an inability to control intake, intense alcohol-seeking, and a continuation of use despite significant adverse

personal and societal consequences. The neurobiological foundation of AUD involves complex maladaptations within central neural pathways that orchestrate for processing reward, regulating reward and stress, and exerting executive control—alterations that collectively perpetuate addictive behaviors [2]. Despite ongoing efforts, conventional pharmacological and behavioral interventions frequently demonstrate limited long-term efficacy and high rates of relapse. This treatment gap underscores an urgent need to explore innovative therapeutic strategies that address the multifaceted pathophysiology of addiction [3].

Research has increasingly focused on the gut-brain axis—a sophisticated bidirectional signaling network that tightly couples enteric and central nervous system activity—as a promising new frontier for therapeutic development [4]. Within this axis, the gut microbiota serves as a critical modulator, directly influencing brain function and subsequent behavior. Chronic excessive alcohol intake is known to disrupt this delicate ecosystem, leading to gut dysbiosis, a compromise of intestinal barrier integrity (often termed "leaky gut"), and the promotion of a state of low-grade systemic inflammation [5]. It is hypothesized that this alcohol-induced gastrointestinal disruption fuels central nervous system pathology by exacerbating two key interconnected processes: neuroinflammation and oxidative stress. Consequently, therapeutic strategies aimed at restoring gut homeostasis present a compelling and novel avenue for intervention in AUD [6]. While direct measurement of gut permeability or microbiota was not performed here, this hypothesis guides our mechanistic interpretation

The mechanistic pathway connecting gut dysbiosis to neuropsychiatric and behavioral symptoms in AUD is thought to cause the microbial products including bacterial lipopolysaccharide (LPS), breach the intestinal barrier and enter the systemic circulation at elevated rates. This endotoxemia triggers a robust peripheral immune response, A hallmark of this state is a marked increase in pro-inflammatory mediators, including the cytokines IL-6 and IL-1β [7]. These circulating inflammatory signals can, in turn, activate microglia within the brain, induce a state of neuroinflammation, and disrupt the normative signaling of neurotransmitters within the mesocorticolimbic dopamine system—a circuit fundamental to reward perception, motivation, and the development of addiction [8]. Ethanol metabolism directly produces a surplus of reactive oxygen species (ROS), which depletes key endogenous antioxidant systems—including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). This imbalance leads to oxidative stress, causing structural harm to neuronal lipids, proteins, and DNA [9]. The synergistic interplay of neuroinflammation and oxidative stress is believed to impair synaptic plasticity and neuronal viability in key brain regions like the prefrontal cortex, nucleus accumbens, and amygdala, thereby reinforcing both addictive drug-seeking behaviors and negative emotional states commonly associated with AUD [10].

Probiotics are live microorganisms that, when administered in sufficient quantities, provide a measurable health benefit. They represent a direct therapeutic approach to modulate the gut-brain communication pathway [11]. By promoting a healthier gut microbiota composition, strengthening the intestinal epithelial barrier, and modulating systemic immune responses, probiotic supplementation may reduce endotoxin translocation and dampen the associated inflammatory cascade. It is therefore postulated that probiotics could mitigate the downstream neuroinflammatory and oxidative consequences of chronic alcohol exposure, leading to an amelioration of core behavioral symptoms of AUD [12].

Preclinical rodent models are indispensable for elucidating these potential mechanisms. The intermittent-access two-bottle choice paradigm effectively models the voluntary escalation of alcohol intake and the development of preference observed in human AUD [13]. This model is effectively complemented by the Conditioned Place Preference (CPP) paradigm, which assesses the rewarding and motivational properties of alcohol by measuring an animal's preference for an environment previously paired with its administration [14]. Together, these behavioral tools allow for a comprehensive evaluation of therapeutic interventions, assessing impacts on both consummatory behavior and the conditioned reinforcing value of alcohol [15]. Previous investigations have shown that specific probiotic strains can reduce alcohol consumption, lessen hepatic steatosis, and alleviate anxiety-like behaviors in rodents, pointing to beneficial effects mediated through the gut [16]. However, a significant gap remains in understanding the impact of defined, multi-strain probiotic formulations on the core pathology of AUD—specifically, alcohol seeking and reward processing—while concurrently providing a detailed profile of associated central neurochemical and inflammatory changes [17].

Therefore, this investigation aimed to rigorously assess the impact of a defined multi-strain probiotic ("Ecotec") on alcohol-seeking behaviors using a preclinical rodent model. We aimed to evaluate its influence on voluntary intake, alcohol preference, and reward motivation via CPP. Furthermore, we sought to delineate the accompanying effects on biomarkers of neuroinflammation, oxidative stress, and monoaminergic neurotransmission within key reward-related brain areas. The "Ecotec" formulation comprises a consortium of strains, including *Lactobacillus acidophilus*, *Bifidobacterium species*, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* [18]. Multi-strain probiotics are theorized to offer synergistic advantages over single-

strain preparations by colonizing diverse niches within the gut and providing more comprehensive support for microbial balance and barrier function [19].

We hypothesize that chronic administration of this probiotic will significantly attenuate alcohol intake and preference, as well as reduce the expression of alcohol-induced CPP. We further anticipate that these behavioral improvements will correlate with a restoration of peripheral and central antioxidant enzyme activities (SOD, CAT, GSH-Px), a reduction in pro-inflammatory cytokine levels (IL-6, IL-1β) in plasma and brain tissue, and a normalization of dopamine and serotonin dynamics in the caudate nucleus and nucleus accumbens. Demonstrating this coordinated set of effects would provide robust mechanistic support for targeting the gut-brain axis as a viable therapeutic strategy for AUD.

2. Materials and Methods

2.1. Experimental Animals

This study utilized adult male Albino Wistar rats (initial weight 180–200 g), sourced from the in-house breeding colony at the HEJ Institute of Chemistry, University of Karachi. Animals were housed under standard laboratory conditions (12-h light/dark cycle, 22 ± 2 °C, ad libitum access to standard chow) in accordance with institutional guidelines [20]. All experimental procedures were reviewed and approved by the Institutional Bioethics Committee (Approval No. IBC-KU-712/2025).

2.2. Pharmacological Agents and Administration

Probiotic: The commercially available multi-strain probiotic formulation "Ecotec" was used, containing *Lactobacillus acidophilus, Bifidobacterium spp., Streptococcus thermophilus,* and *Lactobacillus delbrueckii*. It was administered orally (via oral gavage) at a daily dose of 10^9 colony-forming units (CFU)/mL/kg [21].

Ethanol: A chronic intermittent ethanol exposure model was employed. Ethanol (Merck, Darmstadt, Germany) was presented as the sole fluid source in graduated drinking bottles. To facilitate acclimation, rats were initially given a 2% (*v*/*v*) ethanol solution for two days, followed by a 5% (*v*/*v*) solution for the next two days. For the remainder of the 5-week study period, animals received a 10% (*v*/*v*) ethanol solution [22]. Control animals received plain tap water.

2.3. Study Design and Group Allocation

A total of forty-eight (n = 48) rats were randomly divided into four experimental groups (n = 12 per group):

(1) Water-Control (WC): Received tap water only.
(2) Water-Probiotic (WP): Received tap water supplemented with the probiotic.
(3) Ethanol-Control (EC): Received the escalating ethanol solution.
(4) Ethanol-Probiotic (EP): Received the escalating ethanol solution supplemented with the probiotic.

Treatments were administered daily for a duration of five weeks. Behavioral assessments began on day 0 (baseline) and concluded with a final test on day 35. Following the behavioral tests, animals were euthanized by decapitation. Trunk blood was collected for plasma separation, and brain regions of interest were rapidly dissected. All samples were flash-frozen in liquid nitrogen and stored at −80 °C until biochemical and neurochemical analysis (Figure 1).

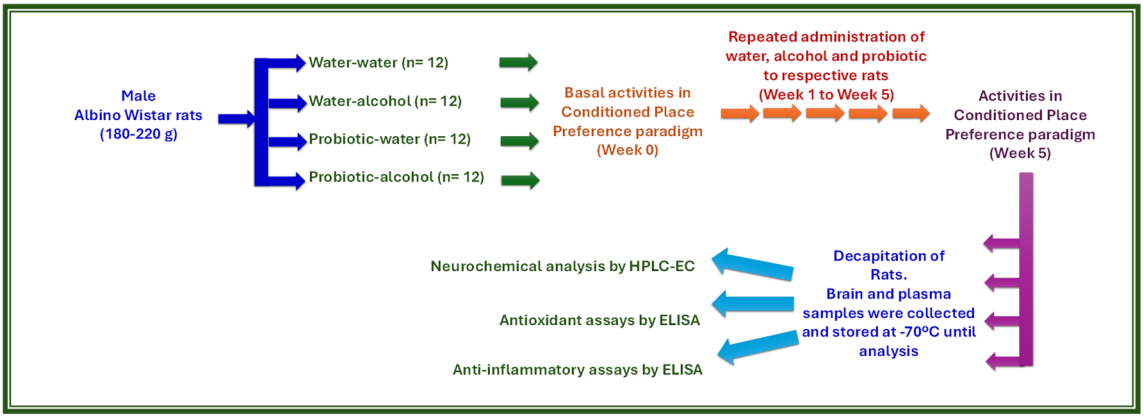


Figure 1. Experimental scheme. Rats were randomly assigned to treatment groups and underwent a baseline Conditioned Place Preference (CPP) test (Week 0), followed by 5 weeks of chronic ethanol and/or probiotic administration. A final CPP test was performed before tissue collection for biochemical and neurochemical analyses.

2.4. Assessment of Drinking Behavior and Motivation

2.4.1. Ethanol Consumption and Preference

Fluid consumption was monitored daily by weighing the drinking bottles. Ethanol intake and preference were calculated weekly using standard formulas [23]:

$$\text{Ethanol Preference (\%)} = [\text{Volume of Ethanol Consumed (mL)}/\text{Total Fluid Volume Consumed (mL)}] \times 100$$

$$\text{Ethanol Intake (g/kg/day)} = [\text{Ethanol Volume (mL/day)} \times \text{Ethanol Concentration (0.1)} \times \text{Density of Ethanol (0.789 g/mL)}]/\text{Body Weight (kg)}$$

2.4.2. Conditioned Place Preference (CPP)

The rewarding properties of ethanol were evaluated using a three-chamber CPP apparatus, as previously described in our laboratory [24]. The apparatus consisted of two large, distinct conditioning compartments (differing in wall pattern and floor texture) connected by a smaller neutral central chamber.

Pre-conditioning (Day 0): Rats were allowed to freely explore all three chambers for 15 min. The time spent in each compartment was recorded to confirm the absence of innate bias.

Conditioning (Days 1–12): Over 12 days, animals underwent a standard conditioning protocol. On alternating days, they were confined for 30 min to one compartment immediately following administration of their assigned fluid (ethanol or water). The other compartment was paired with the alternative fluid.

Post-conditioning Test (Day 13): Animals were again placed in the neutral chamber with free access to all compartments for 15 min. The time spent in the ethanol-paired versus water-paired compartment was video-recorded and analyzed. An increased preference for the ethanol-paired context indicates a learned rewarding association.

2.5. Biochemical Analyses

2.5.1. Evaluation of Antioxidant Enzyme Activities

The activities of key antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px)—were measured in plasma and brain tissue homogenates using established spectrophotometric assays [24]. SOD activity was determined by monitoring the inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm. CAT activity was assayed by measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm. GSH-Px activity was quantified by coupling the oxidation of glutathione (GSH) to the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), with absorbance read at 412 nm. Enzyme activities were expressed in standard international units.

2.5.2. Quantification of Inflammatory Markers

The concentrations of the pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-1β (IL-1β), as well as Brain-Derived Neurotrophic Factor (BDNF), were measured in plasma and brain homogenate supernatants. Commercially available, rat-specific enzyme-linked immunosorbent assay (ELISA) kits (Merck KGaA, Darmstadt, Germany) were used according to the manufacturer's detailed protocols [25]. Sample concentrations were interpolated from a standard curve generated with each assay run at respective wavelengths for IL-6 (450 nm and 630 nm), IL-1β (450 nm) and BDNF (450 nm).

2.6. Brain Tissue Processing and Neurochemical Analysis

2.6.1. Microdissection

On the day of sacrifice, brains were rapidly extracted, rinsed in ice-cold saline, and placed on a chilled brain matrix. Using established anatomical landmarks [26], the caudate putamen and nucleus accumbens were carefully dissected bilaterally with fine surgical tools on an ice-cold plate. Tissues were immediately weighed, flash-frozen, and stored at −80 °C.

2.6.2. High-Performance Liquid Chromatography with Electrochemical Detection (HPLC-EC)

Monoamine and metabolite levels (dopamine, DOPAC, HVA, serotonin, 5-HIAA) were quantified using HPLC-EC, following a well-characterized extraction and analysis protocol ([27], modified). Briefly, brain tissue

samples were homogenized in a 5× volume of chilled 0.1 M perchloric acid containing an internal standard. The homogenates were centrifuged at high speed (10,000× *g* for 15 min at 4 °C), and the resulting supernatants were filtered. Chromatographic separation was achieved using a reversed-phase C18 column (Shim-pack ODS-18, 250 × 4.6 mm, 5 µm). The mobile phase consisted of 0.1 M sodium phosphate buffer (pH 2.9), 14% methanol, 0.023% octyl sodium sulfate, and 0.0035% EDTA, delivered isocratically at a flow rate of 1.0 mL/min. Detection was performed with an electrochemical detector (LEC 6A, Schimadzu, Kyoto, Japan) with the working electrode potential set at +0.8 V versus an Ag/AgCl reference. Analyte concentrations were calculated by comparing peak areas to those of external standards.

2.7. Statistical Analysis

All quantitative data are presented as the mean ± standard deviation (SD). Behavioral data over time were analyzed using two-way or three-way Analysis of Variance (ANOVA) with repeated measures, where appropriate, followed by Tukey's post-hoc test for multiple comparisons. Biochemical and neurochemical data from the endpoint were analyzed using two-way ANOVA (factors: Ethanol and Probiotic). All statistical analyses were performed using SPSS software ver 25.0 (IBM Corp, Armonk, NY, USA). A probability value of $p < 0.05$ was considered statistically significant for all tests.

3. Results

Figure 2 shows effects of probiotic supplementation in ethanol treated rats on fluid intake. Data analysis results by three-way ANOVA are shown in Table 1.

Table 1. Effects of probiotic supplementation in ethanol treated rats on fluid intake.

	df	F	*p* Value
Week 0 (Figure 2a)			
Fluid intake	1, 88	65.02	0.001
Probiotic	1, 88	1.25	0.21
Ethanol	1, 88	2.08	0.14
Ethanol*probiotic	1, 88	1.05	0.45
Ethanol*fluid intake	1, 88	1.41	0.50
Probiotic*fluid intake	1, 88	1.49	0.68
Ethanol*probiotic*fluid intake	1, 88	1.71	0.15
Week 1 (Figure 2b)			
Fluid intake	1, 88	48.26	0.001
Probiotic	1, 88	1.65	0.59
Ethanol	1, 88	25.01	0.001
Ethanol*probiotic	1, 88	56.18	0.001
Ethanol*fluid intake	1, 88	52.15	0.001
Probiotic*fluid intake	1, 88	1.02	0.41
Ethanol*probiotic*fluid intake	1, 88	2.05	0.59
Week 2 (Figure 2c)			
Fluid intake	1, 88	25.95	0.001
Probiotic	1, 88	2.05	0.74
Ethanol	1, 88	52.03	0.001
Ethanol*probiotic	1, 88	30.25	0.001
Ethanol*fluid intake	1, 88	24.08	0.001
Probiotic*fluid intake	1, 88	1.45	0.73
Ethanol*probiotic*fluid intake	1, 88	1.09	0.25
Week 3 (Figure 2d)			
Fluid intake	1, 88	35.02	0.001
Probiotic	1, 88	2.40	0.54
Ethanol	1, 88	32.08	0.001
Ethanol*probiotic	1, 88	31.47	0.001
Ethanol*fluid intake	1, 88	20.48	0.001
Probiotic*fluid intake	1, 88	1.05	0.41
Ethanol*probiotic*fluid intake	1, 88	1.76	0.65

Table 1. *Cont.*

	df	F	*p* Value
Week 4 (Figure 2e)			
Fluid intake	1, 88	40.25	0.001
Probiotic	1, 88	1.85	0.63
Ethanol	1, 88	48.21	0.001
Ethanol*probiotic	1, 88	35.07	0.001
Ethanol*fluid intake	1, 88	20.65	0.001
Probiotic*fluid intake	1, 88	2.05	0.71
Ethanol*probiotic*fluid intake	1, 88	1.54	0.65
Week 5 (Figure 2f)			
Fluid intake	1, 88	45.03	0.001
Probiotic	1, 88	1.05	0.54
Ethanol	1, 88	51.05	0.001
Ethanol*probiotic	1, 88	41.05	0.001
Ethanol*fluid intake	1, 88	20.15	0.001
Probiotic*fluid intake	1, 88	1.85	0.54
Ethanol*probiotic*fluid intake	1, 88	1.47	0.32

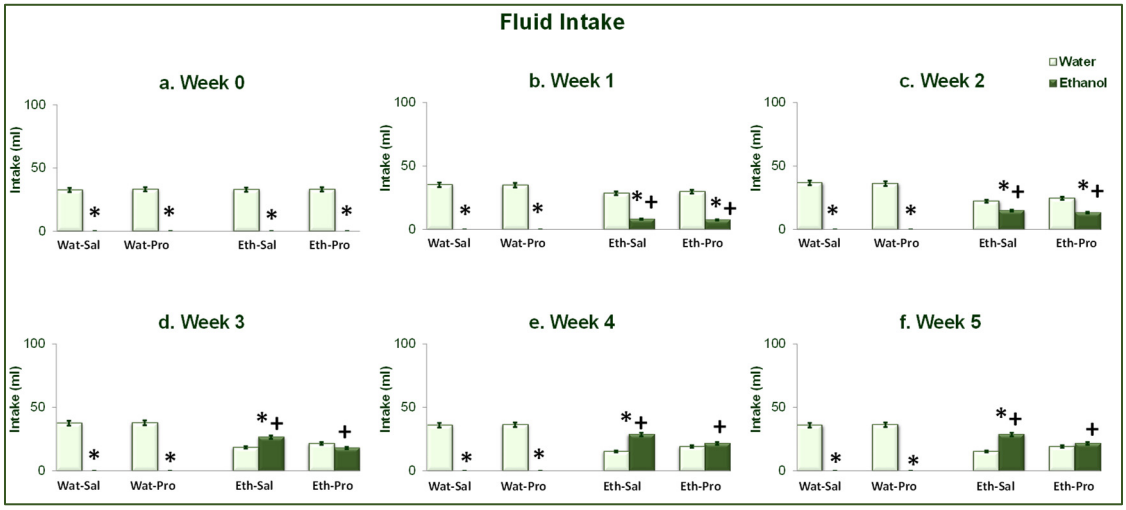


Figure 2. Effects of probiotic supplementation in ethanol treated rats on fluid intake. Values are means ± SD (n = 12). Significant differences by Tukey's test: * $p < 0.01$ as compared to respective water intake; + $p < 0.01$ as compared to respective water treated rats following three-way ANOVA.

Figure 3 shows effects of probiotic supplementation in ethanol treated rats on ethanol preference. Data analysis results by two-way/three-way ANOVA are shown in Table 2.

Table 2. Effects of probiotic supplementation in ethanol treated rats on ethanol preference.

	df	F	*p* Value
% ethanol preference (Figure 3a)			
Treatments	1, 132	38.59	0.001
Repeated monitoring	1, 132	52.36	0.001
Treatments*repeated monitoring	1, 132	41.65	0.001
Ethanol intake in g/kg/day (Figure 3b)			
Treatments	1, 132	65.02	0.001
Repeated monitoring	1, 132	48.01	0.001
Treatments*repeated monitoring	1, 132	53.09	0.001
Place preference on week 0 (Figure 3c)			
Ethanol	1, 132	1.25	0.38
Probiotic	1, 132	56.16	0.62
Compartments	1, 132	1.60	0.74
Ethanol*probiotic	1, 132	1.85	0.41
Ethanol*compartments	1, 132	2.05	0.54
Probiotic*compartments	1, 132	1.85	0.55
Ethanol*probiotic*compartments	1, 132	0.56	0.85

Table 2. *Cont.*

	df	F	*p* Value
Place preference on week 5 (Figure 3d)			
Ethanol	1, 132	32.25	0.001
Probiotic	1, 132	32.85	0.001
Compartments	1, 132	64.14	0.001
Ethanol*probiotic	1, 132	31.25	0.001
Ethanol*compartments	1, 132	28.09	0.001
Probiotic*compartments	1, 132	62.14	0.001
Ethanol*probiotic*compartments	1, 132	54.08	0.001

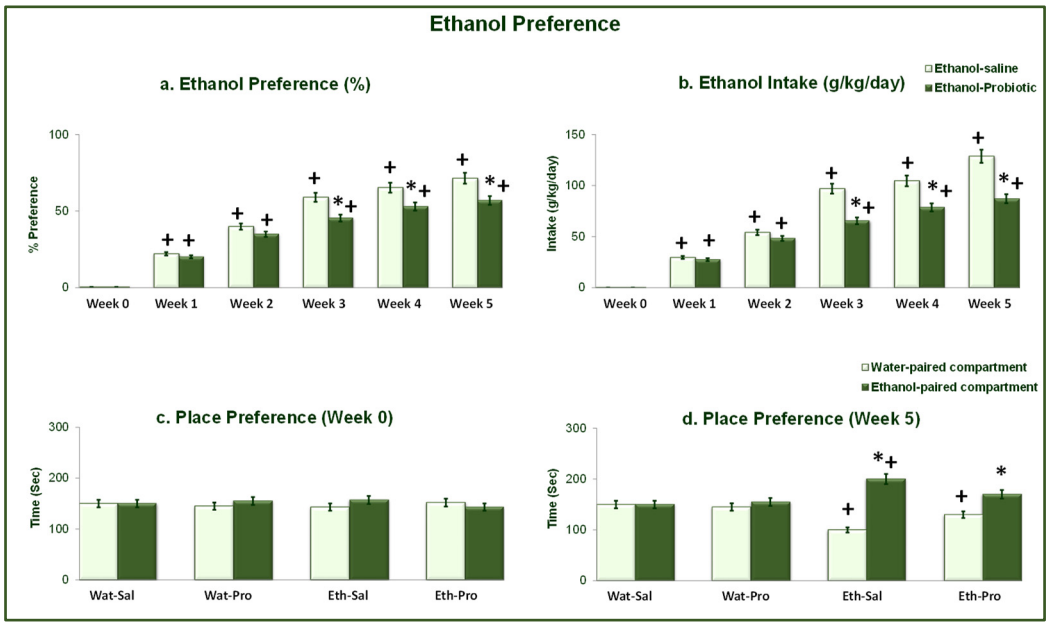


Figure 3. Effects of probiotic supplementation in ethanol treated rats on ethanol preference. Values are means ± SD ($n = 12$). Significant differences by Tukey's test: (**a**,**b**): * $p < 0.01$ as compared to respective ethanol-saline treated rats; + $p < 0.01$ as compared to respective week 0 values following two-way ANOVA. (**c**,**d**): * $p < 0.01$ as compared to respective water-paired compartment; + $p < 0.01$ as compared to respective water treated rats following three-way ANOVA.

Figure 4 shows effects of probiotic supplementation in ethanol treated rats on antioxidant profile. Data analysis results by two-way ANOVA are shown in Table 3.

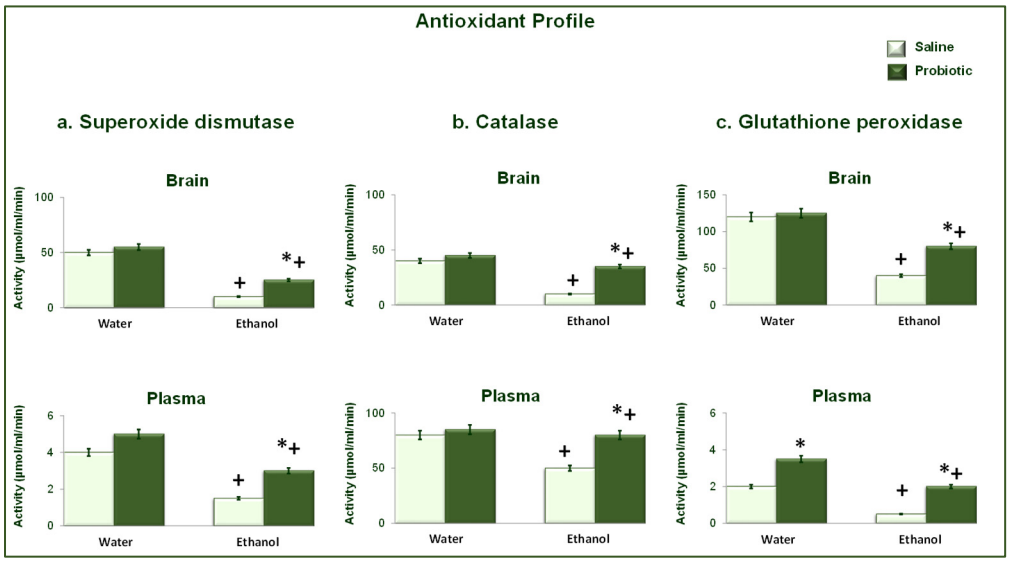


Figure 4. Effects of probiotic supplementation in ethanol treated rats on antioxidant profile. Values are means ± SD ($n = 12$). Significant differences by Tukey's test: * $p < 0.01$ as compared to respective saline treated rats; + $p < 0.01$ as compared to respective water treated rats following two-way ANOVA.

Table 3. Effects of probiotic supplementation in ethanol treated rats on antioxidant profile.

	df	F	*p* Value
Superoxide dismutase activity in brain (Figure 4a)			
Ethanol	1, 44	45.02	0.001
Probiotic	1, 44	38.65	0.001
Ethanol*probiotic	1, 44	74.02	0.001
Superoxide dismutase activity in plasma (Figure 4a)			
Ethanol	1, 44	38.06	0.001
Probiotic	1, 44	42.59	0.001
Ethanol*probiotic	1, 44	38.79	0.001
Catalase activity in brain (Figure 4b)			
Ethanol	1, 44	61.08	0.001
Probiotic	1, 44	34.16	0.001
Ethanol*probiotic	1, 44	59.16	0.001
Catalase activity in plasma (Figure 4b)			
Ethanol	1, 44	23.15	0.001
Probiotic	1, 44	45.02	0.001
Ethanol*probiotic	1, 44	38.41	0.001
Glutathione peroxidase activity in brain (Figure 4c)			
Ethanol	1, 44	46.34	0.001
Probiotic	1, 44	51.69	0.001
Ethanol*probiotic	1, 44	47.05	0.001
Glutathione peroxidase activity in plasma (Figure 4c)			
Ethanol	1, 44	32.08	0.001
Probiotic	1, 44	21.16	0.001
Ethanol*probiotic	1, 44	41.85	0.001

Figure 5 shows effects of probiotic supplementation in ethanol treated rats on inflammatory profile. Data analysis results by two-way ANOVA are shown in Table 4.

Table 4. Effects of probiotic supplementation in ethanol treated rats on inflammatory profile.

	df	F	*p* Value
IL-6 levels in brain (Figure 5a)			
Ethanol	1, 44	26.18	0.001
Probiotic	1, 44	20.89	0.001
Ethanol*probiotic	1, 44	31.08	0.001
IL-6 levels in plasma (Figure 5a)			
Ethanol	1, 44	32.54	0.001
Probiotic	1, 44	16.54	0.001
Ethanol*probiotic	1, 44	28.63	0.001
IL-1β levels in brain (Figure 5b)			
Ethanol	1, 44	32.14	0.001
Probiotic	1, 44	28.65	0.001
Ethanol*probiotic	1, 44	35.68	0.001
IL-1β levels in plasma (Figure 5b)			
Ethanol	1, 44	27.15	0.001
Probiotic	1, 44	23.98	0.001
Ethanol*probiotic	1, 44	31.05	0.001
BDNF levels in brain (Figure 5c)			
Ethanol	1, 44	23.58	0.001
Probiotic	1, 44	42.05	0.001
Ethanol*probiotic	1, 44	36.02	0.001
BDNF levels in plasma (Figure 5c)			
Ethanol	1, 44	41.58	0.001
Probiotic	1, 44	26.13	0.001
Ethanol*probiotic	1, 44	28.57	0.001

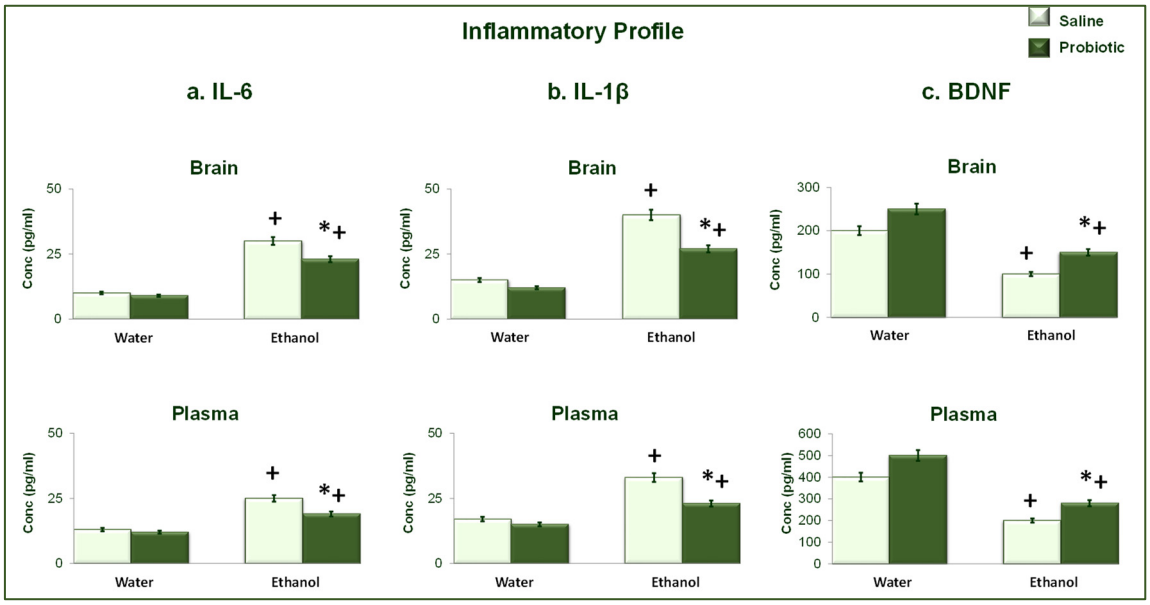


Figure 5. Effects of probiotic supplementation in ethanol treated rats on inflammatory profile. Values are means ± SD (n = 12). Significant differences by Tukey's test: * $p < 0.01$ as compared to respective saline treated rats; + $p < 0.01$ as compared to respective water treated rats following two-way ANOVA.

Figure 6 shows effects of probiotic supplementation in ethanol treated rats on neurochemical profile. Data analysis results by two-way ANOVA are shown in Table 5.

Table 5. Effects of probiotic supplementation in ethanol treated rats on neurochemical profile (df = 1, 44; p = 0.001).

	Dopamine	DOPAC	HVA	5HT	5HIAA
		Caudate (Figure 6a)			
Ethanol	62.58	25.35	46.05	54.62	52.13
Probiotic	75.05	35.04	38.46	64.25	47.92
Ethanol*probiotic	65.36	30.54	56.92	39.01	65.83
		Nucleus accumbens (Figure 6b)			
Ethanol	45.08	35.06	52.36	65.03	45.26
Probiotic	53.16	45.03	48.09	85.05	35.16
Ethanol*probiotic	35.64	62.01	32.65	75.43	65.01

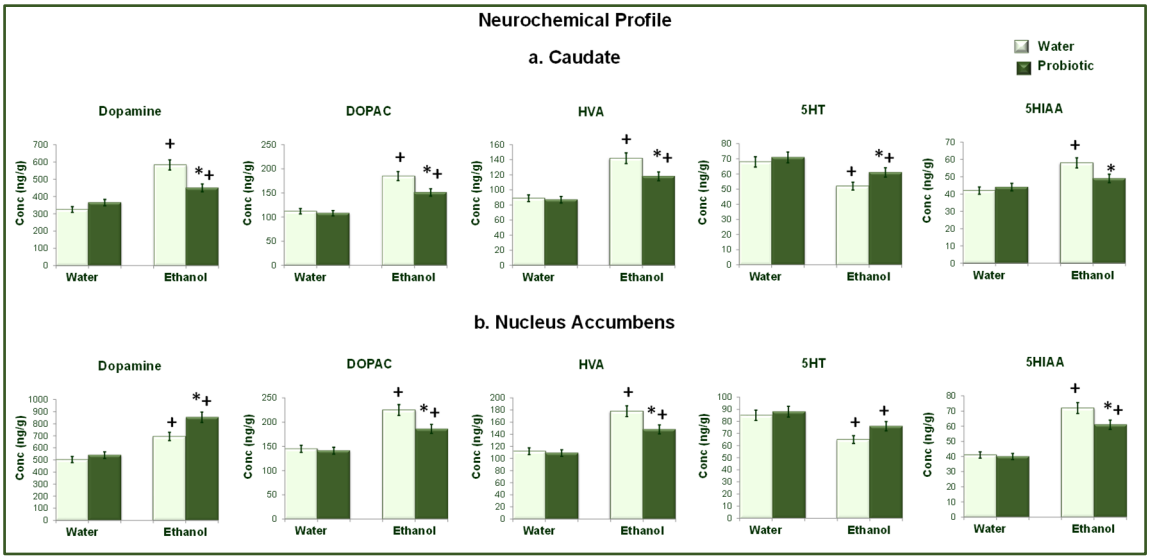


Figure 6. Effects of probiotic supplementation in ethanol treated rats on antioxidant profile. Values are means ± SD (n = 12). Significant differences by Tukey's test: * $p < 0.01$ as compared to respective saline treated rats; + $p < 0.01$ as compared to respective water treated rats following two-way ANOVA.

4. Discussion

This study provides integrated evidence that chronic administration of the multi-strain probiotic "Ecotec" effectively mitigates core behavioral features of Alcohol Use Disorder in a rodent model. Our findings demonstrate that probiotic co-administration not only reduced voluntary ethanol consumption and preference over a sustained five-week period (Figure 3a,b) but also attenuated the expression of ethanol-induced conditioned place preference (Figure 3d). These results indicate a significant reduction in both the consummatory and the conditioned rewarding properties of alcohol. These behavioral improvements align with and extend prior research documenting that specific probiotic strains, such as *Lactobacillus rhamnosus*, can diminish voluntary alcohol intake and associated hepatic pathology [28]. The delayed onset of behavioral efficacy, emerging clearly from the third week onward (Figure 3a,b), suggests a time-dependent therapeutic mechanism. This likely reflects the period required for probiotic colonization, gut microbiota modulation, and the subsequent induction of systemic effects [29]. While direct measures of gut colonization were not performed in this study, future work should include fecal microbial analysis to confirm probiotic establishment. The concurrent attenuation of home cage drinking and cue-driven reward-seeking provides robust, multi-faceted support for the potential of probiotics to modify fundamental components of addiction beyond simple consumption metrics [30]. Furthermore, probiotic treatment significantly increased Brain-Derived Neurotrophic Factor (BDNF) levels in both plasma and brain tissues (Figure 5), which were suppressed by chronic ethanol exposure. The restoration of BDNF is particularly noteworthy, as BDNF is a critical mediator of synaptic plasticity, neurogenesis, and cognitive function, and its depletion is implicated in the pathophysiology of addictive behaviors and neurotoxicity associated with chronic alcohol use. The elevation of BDNF following probiotic supplementation suggests an enhancement of neurotrophic support and adaptive plasticity, which may underlie the observed behavioral recovery and neurochemical stabilization.

The robust anti-inflammatory effects we observed offer a compelling mechanistic link between probiotic action and behavioral recovery. Consistent with the established role of ethanol in promoting inflammation [31], chronic exposure in our model significantly elevated levels of the pro-inflammatory cytokines IL-6 and IL-1β in both the periphery and the brain (Figure 5). Probiotic treatment successfully countered this increase (Figure 5a,b). This finding supports the hypothesis that by restoring gut barrier integrity, the probiotic formulation reduced the translocation of bacterial endotoxins into circulation, thereby dampening the activation of the innate immune system [32]. Direct confirmation of reduced gut permeability and endotoxin translocation (e.g., via plasma LPS or FITC-dextran assay) was not obtained in this study; therefore, the proposed gut-restorative mechanism remains speculative and requires future investigation. Future studies involving direct measures of gut permeability (e.g., plasma LPS, FITC-dextran assay) would strengthen this mechanistic link, and such assays are recommended for future investigations. The attenuation of both peripheral and central cytokine levels is critically important, as neuroinflammation is a key driver of synaptic dysfunction, neuronal damage, and the reinforcement of maladaptive behaviors within the mesocorticolimbic reward pathway [33]. The capacity of this specific probiotic consortium to suppress inflammatory markers substantiates the premise that a primary mechanism of its benefit is the inhibition of the gut-derived inflammatory cascade, offering protection against alcohol-induced neuroimmunopathology [34,35]. This anti-inflammatory milieu likely contributes to behavioral recovery by creating a less permissive neural environment for the development and persistence of addiction-related neuroplasticity.

Our data further reveal that probiotic supplementation powerfully reversed the alcohol-induced suppression of crucial endogenous antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px)—in both plasma and brain tissue (Figure 4). Chronic ethanol consumption is a potent inducer of oxidative stress, generating reactive oxygen species that deplete antioxidant reserves and lead to neuronal lipid peroxidation, protein damage, and DNA injury [36]. The restoration of these enzymatic defenses by probiotic treatment (Figure 4a–c) indicates an enhanced capacity to neutralize oxidative insults. This effect may be mediated through the modulation of gut-derived metabolites that influence host antioxidant gene expression or via the reduction of primary inflammatory drivers that exacerbate oxidative stress [37]. This finding is pivotal, as oxidative stress acts in synergy with neuroinflammation to impair mitochondrial function and promote neuronal apoptosis in key regions such as the nucleus accumbens and prefrontal cortex [38,39]. By fortifying the brain's endogenous antioxidant systems, probiotic treatment may help preserve neuronal integrity and synaptic function, thereby contributing to normalized behavior and representing a second, interrelated pathway for its therapeutic efficacy.

Neurochemical analyses indicated that probiotic treatment partially restored the alcohol-induced dysregulation of dopaminergic and serotonergic transmission in the caudate nucleus and nucleus accumbens (Figure 6). As anticipated, chronic ethanol exposure elevated levels of dopamine and its metabolites (DOPAC, HVA) in these regions (Figure 6a,b), reflective of a hyperdopaminergic state linked to reward sensitization and increased incentive salience [40]. Probiotic administration significantly attenuated this neurochemical elevation,

suggesting a normalization of dopaminergic hyperactivity. Concomitantly, the probiotic also mitigated the alcohol-induced rise in the serotonin metabolite 5-HIAA (Figure 6a,b), which is frequently associated with heightened serotonin turnover and anxiety-like states [41]. It should be noted that probiotic treatment further reduced 5-HT levels in some regions, indicating region-specific modulation of serotonergic activity that warrants further investigation into underlying receptor or turnover mechanisms. The modulation of these key neurotransmitter systems is likely a downstream consequence of the probiotic's foundational anti-inflammatory and antioxidant actions, as both inflammation and oxidative stress can directly influence the activity of tyrosine hydroxylase and tryptophan hydroxylase, the rate-limiting enzymes for dopamine and serotonin synthesis, respectively [42,43]. By promoting a more balanced neurochemical environment within critical reward and affect-regulation circuits, the probiotic may reduce both the hyper-rewarding effects of alcohol and the negative reinforcement associated with withdrawal, thereby decreasing the overall motivation to consume alcohol.

5. Conclusions

In summary, the findings from this preclinical investigation provide convergent evidence that supplementation with the multi-strain probiotic formulation Ecotec alleviates alcohol-seeking behaviors through a multi-mechanistic action potentially involving the gut-brain axis. The observed reduction in alcohol seeking and reward-related motivation correlates strongly with the attenuation of systemic and neuroinflammation, the enhancement of endogenous antioxidant defenses, and a partial restoration of normative monoaminergic signaling in key brain regions. These results underscore the therapeutic promise of targeting the gut microbiome as a complementary strategy in the management of Alcohol Use Disorder. They further reinforce the broader principle that interventions designed to reestablish gut eubiosis can exert profound, beneficial effects on central nervous system function and maladaptive behaviors in the context of substance addiction. Future research should prioritize the translation of these encouraging preclinical results into controlled clinical trials to evaluate the efficacy and safety of specific probiotic regimens in individuals with alcohol dependence.

6. Limitations

Despite the promising findings, several limitations of this study should be acknowledged. First, while our data support a gut-brain axis mechanism, we did not directly assess gut microbiota composition, intestinal permeability, or systemic endotoxin levels. Future studies incorporating 16S rRNA sequencing, FITC-dextran assays, or plasma LPS measurements would provide more direct evidence of probiotic-mediated gut restoration. Second, the study was conducted exclusively in male rats; thus, potential sex differences in the probiotic response remain unexplored. Third, this remains a preclinical investigation, and the translation of these results to clinical populations with Alcohol Use Disorder requires validation through controlled human trials. Finally, the probiotic formulation was used as a whole consortium; determining the contributions of individual strains to the observed effects would further refine future therapeutic applications.

Author Contributions

H.I.: conceptualization, methodology, software, data curation, writing—original draft preparation. supervision; S.P.: visualization, investigation; D.J.H.: writing—reviewing and editing. All authors have read and agreed to the publishing of the manuscript.

Institutional Review Board Statement

All protocols were approved by Institutional Bioethics Committee (Approval No. IBC-KU-215/2025).

Informed Consent Statement

Not applicable.

Data Availability Statement

Data would be available upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies

No AI tools were used during the preparation of this manuscript. The authors take full responsibility for the content of the published article.

References

1. Cui, X.; Liu, K.; Ji, Y.; et al. Global trends in the burden of alcohol use disorders in the working-age population from 1990 to 2021 and projections for the next 20 years. *Front. Public Health* **2025**, 13, 1616343.
2. Yang, W.; Singla, R.; Maheshwari, O.; et al. Alcohol Use Disorder: Neurobiology and Therapeutics. *Biomedicines* **2022**, 10, 1192.
3. Alorfi, N.M. Investigating Therapeutic Targets for Alcohol Use Disorder: Pharmacological View of ClinicalTrials.gov Data. *Int. J. Gen. Med.* **2025**, 18, 3701–3712.
4. Shukla, S.; Hsu, C.L. Alcohol Use Disorder and the Gut-Brain Axis: A Narrative Review of the Role of Gut Microbiota and Implications for Treatment. *Microorganisms* **2025**, 13, 67.
5. Pich, E.M.; Tarnanas, I.; Brigidi, P.; et al. Gut Microbiome-Liver-Brain axis in Alcohol Use Disorder. The role of gut dysbiosis and stress in alcohol-related cognitive impairment progression: Possible therapeutic approaches. *Neurobiol. Stress* **2025**, 35, 100713.
6. Dong, Y.; Buczynski, M.W.; Gregus, A.M. Alcohol use disorder-associated pain: Clinical and preclinical evidence. *Alcohol* **2025**, 129, 14–40.
7. Koutromanos, I.; Legaki, E.; Dovrolis, N.; et al. Integrating gut microbiome and neuroplasticity genomics in alcohol use disorder therapy. *Hum. Genom.* **2025**, 19, 78.
8. Erickson, E.K.; Grantham, E.K.; Warden, A.S.; et al. Neuroimmune signaling in alcohol use disorder. *Pharmacol. Biochem. Behav.* **2019**, 177, 34–60.
9. Tsermpini, E.E.; Plemenitaš Ilješ, A.; Dolžan, V. Alcohol-Induced Oxidative Stress and the Role of Antioxidants in Alcohol Use Disorder: A Systematic Review. *Antioxidants* **2022**, 11, 1374.
10. Avchalumov, Y.; Mandyam, C.D. Synaptic Plasticity and its Modulation by Alcohol. *Brain Plast.* **2020**, 6, 103–111.
11. Pisarello, M.J.L.; Marquez, A.; Chaia, A.P.; et al. Targeting gut health: Probiotics as promising therapeutics in alcohol-related liver disease management. *AIMS Microbiol.* **2025**, 11, 410–435.
12. Shukla, P.K.; Meena, A.S.; Rao, R. Prevention and mitigation of alcohol-induced neuroinflammation by Lactobacillus plantarum by an EGF receptor-dependent mechanism. *Nutr. Neurosci.* **2022**, 25, 871–883.
13. Hou, S.; Arce Soto, N.M.; Glover, E.J. Modeling Alcohol Consumption in Rodents Using Two-Bottle Choice Home Cage Drinking and Microstructural Analysis. *J. Vis. Exp.* **2024**, *213*, e67486. https://doi.org/10.3791/67486.
14. Sklenarik, S.M.; Burrows, C.N.; Astur, R.S. Conditioned place preferences for virtual alcohol cues. *Behav. Brain Res.* **2023**, 438, 114176.
15. Hallihan, H.; Srimoragot, M.; Ma, J.; et al. Integrated behavioral interventions for adults with alcohol use disorder: A systematic review. *Drug Alcohol Depend.* **2024**, 263, 111406.
16. Vidya Bernhardt, G.; Shivappa, P.; Pinto, J.R.; et al. Probiotics-role in alleviating the impact of alcohol liver disease and alcohol deaddiction: A systematic review. *Front. Nutr.* **2024**, 11, 1372755.
17. Fuenzalida, C.; Dufeu, M.S.; Poniachik, J.; et al. Probiotics-Based Treatment as an Integral Approach for Alcohol Use Disorder in Alcoholic Liver Disease. *Front. Pharmacol.* **2021**, 12, 729950.
18. Bettocchi, S.; Comotti, A.; Elli, M.; et al. Probiotics and Fever Duration in Children With Upper Respiratory Tract Infections: A Randomized Clinical Trial. *JAMA Netw. Open* **2025**, *8*, e250669.
19. Kwoji, I.D.; Aiyegoro, O.A.; Okpeku, M.; et al. Multi-Strain Probiotics: Synergy among Isolates Enhances Biological Activities. *Biology* **2021**, 10, 322.
20. Ikram, H.; Atique, I.; Moosa, U.M.; et al. Mitigation of addictive effects induced by lorazepam through concurrent administration of SSRI: Interplay of serotonin and dopamine in cAUDate and nucleus accumbens. *Pak. J. Pharm. Sci.* **2024**, 37, 1043–1051.
21. Wang, Y.; Kirpich, I.; Liu, Y.; et al. Lactobacillus rhamnosus GG treatment potentiates intestinal hypoxia-inducible factor, promotes intestinal integrity and ameliorates alcohol-induced liver injury. *Am. J. Pathol.* **2011**, 179, 2866–2875.
22. Haleem, D.J.; Naz, H.; Parveen, T.; et al. Serotonin and serotonin 1-A receptors in the failure of ethanol-treated rats to adapt to a repeated stress schedule. *J. Stud. Alcohol* **2002**, 63, 389–396.
23. Velten Mendes, M.; Lima, T.C.; Amormino, M.S.; et al. Predicted functional alterations in colonic microbiota metabolism underlie ethanol consumption and preference behavior in mice. *Alcohol Clin. Exp. Res.* **2025**, 49, 2436–2450.
24. Ikram, H.; Zakir, R.; Haleem, D.J. Memory enhancing and neuroprotective effects of apomorphine in a rat model of dementia. *Metab. Brain Dis.* **2024**, 39, 1051–1063.

25. Ikram, H.; Perveen, S.; Haleem, D.J. Dual role of dietary tryptophan on memory regulation in male albino wistar rats—Synergistic modulation of serotonergic signaling and kynurenine pathway. *Nutr. Neurosci.* **2026**, *29*, 356–370.
26. Ikram, H.; Haleem, D.J. Regional neurochemical profile following coadministration of apomorphine and buspirone in an animal model of addiction. *Pak. J. Pharm. Sci.* **2025**, 38, 2168–2175.
27. Ikram, H.; Shahla, P.; Ali, S.; et al. Reduced 5-HT_{2C} receptor responsiveness in rats after chronic midazolam treatment: Behavioral and neurochemical evidence in rats. *Neurophysiology* **2023**, *55*, 50–60. https://doi.org/10.1007/s11062-025-09954-0.
28. Gupta, H.; Kim, S.H.; Kim, S.K.; et al. Beneficial Shifts in Gut Microbiota by Lacticaseibacillus rhamnosus R0011 and Lactobacillus helveticus R0052 in Alcoholic Hepatitis. *Microorganisms* **2022**, 10, 1474.
29. Li, Y.; Yao, Y.; Zhang, Z.; et al. Research status and development trends of probiotics in clinical applications. *Front. Nutr.* **2025**, 12, 1650883.
30. Winkler, G.A.; Grahame, N.J. Home cage voluntary alcohol consumption increases binge drinking without affecting abstinence-related depressive-like behaviors or operant responding in crossed high alcohol-preferring mice (cHAPs). *Alcohol* **2024**, 116, 9–19.
31. Pei, J.; Zhang, C.; Zhang, Q.; et al. Probiotics alleviate chronic ethanol exposure-induced anxiety-like behavior and hippocampal neuroinflammation in male mice through gut microbiota-derived extracellular vesicles. *J. Nanobiotechnol.* **2024**, 22, 730.
32. Robles-Vera, I.; Jarit-Cabanillas, A.; Brandi, P.; et al. Microbiota translocation following intestinal barrier disruption promotes Mincle-mediated training of myeloid progenitors in the bone marrow. *Immunity* **2025**, 58, 381–396.e9.
33. Escobar, A.P.; Bonansco, C.; Cruz, G.; et al. Central and Peripheral Inflammation: A Common Factor Causing Addictive and Neurological Disorders and Aging-Related Pathologies. *Int. J. Mol. Sci.* **2023**, 24, 10083.
34. Ahmadi-Khorram, M.; Hatami, A.; Asghari, P.; et al. Probiotics mitigate stress and inflammation in malnourished adults via gut microbiota modulation: A randomized controlled trial. *Front. Nutr.* **2025**, *12*, 1615607.
35. Lee, C.; Kim, S.W.; Verma, R.; et al. Probiotic Consortium Confers Synergistic Anti-Inflammatory Effects in Inflammatory Disorders. *Nutrients* **2024**, *16*, 790.
36. Lee, J.Y.; Jee, Y.M.; Yang, K.; et al. Alcohol-Induced Oxidative Stress and Gut-Liver-Brain Crosstalk: Expanding the Paradigm from ALD to MetALD. *Antioxidants* **2025**, *14*, 1196.
37. Tsao, S.P.; Nurrahma, B.A.; Kumar, R.; et al. Probiotic Enhancement of Antioxidant Capacity and Alterations of Gut Microbiota Composition in 6-Hydroxydopamin-Induced Parkinson's Disease Rats. *Antioxidants* **2021**, *10*, 1823.
38. Weinstock, M. Role of Oxidative Stress and Neuroinflammation in the Etiology of Alzheimer's Disease: Therapeutic Options. *Antioxidants* **2025**, *14*, 769.
39. Gorlova, A.; Svirin, E.; Pavlov, D.; et al. Understanding the Role of Oxidative Stress, Neuroinflammation and Abnormal Myelination in Excessive Aggression Associated with Depression: Recent Input from Mechanistic Studies. *Int. J. Mol. Sci.* **2023**, *24*, 915.
40. Garland, K.C.; Madden, G.J. A Pavlovian, conditioned-reinforcement approach to reducing impulsive choice. *Behav. Process.* **2025**, *228*, 105208.
41. Mkrtchian, A.; Qiu, Z.; Abir, Y.; et al. Differential Associations of Dopamine and Serotonin With Reward and Punishment Processes in Humans: A Systematic Review and Meta-Analysis. *JAMA Psychiatry* **2025**, *82*, 818–829.
42. Vecchio, L.M.; Sullivan, P.; Dunn, A.R.; et al. Enhanced tyrosine hydroxylase activity induces oxidative stress, causes accumulation of autotoxic catecholamine metabolites, and augments amphetamine effects *in vivo*. *J. Neurochem.* **2021**, *158*, 960–979.
43. Shi, C.; Dong, J.; Hui, X.; et al. Production, Mechanisms, and Therapeutic Strategies of Tryptophan Metabolites in CNS Diseases. *Mol. Neurobiol.* **2025**, *63*, 226.