Journal of Neuroinflammation

RESEARCH

Open Access

Nitrate ameliorates alcohol-induced cognitive impairment via oral microbiota

Xiangxue Li[1†], Zhaojun Ni[1†], Weixiong Shi[2†], Kangqing Zhao[1], Yanjie Zhang[1,3], Lina Liu[3], Zhong Wang[1], Jie Chen[1], Zhoulong Yu[1], Xuejiao Gao[1], Ying Qin[4], Jingwen Zhao[4], Wenjuan Peng[4], Jie Shi[5], Thomas R. Kosten[6], Lin Lu[1,5], Lei Su[2*], Yanxue Xue[5,7*] and Hongqiang Sun[1*]

Abstract

Alcohol use is associated with cognitive impairment and dysregulated inflammation. Oral nitrate may benefit cognitive impairment in aging through altering the oral microbiota. Similarly, the beneficial effects of nitrate on alcohol-induced cognitive decline and the roles of the oral microbiota merit investigation. Here we found that nitrate supplementation effectively mitigated cognitive impairment induced by chronic alcohol exposure in mice, reducing both systemic and neuroinflammation. Furthermore, nitrate restored the dysbiosis of the oral microbiota caused by alcohol consumption. Notably, removing the oral microbiota led to a subsequent loss of the beneficial effects of nitrate. Oral microbiota from donor alcohol use disordered humans who had been taking the nitrate intervention were transplanted into germ-free mice which then showed increased cognitive function and reduced neuroinflammation. Finally, we examined 63 alcohol drinkers with varying levels of cognitive impairment and found that lower concentrations of nitrate metabolism-related bacteria were associated with higher cognitive impairment and lower nitrate levels in plasma. These findings highlight the protective role of nitrate against alcohol-induced cognition impairment and neuroinflammation and suggest that the oral microbiota associated with nitrate metabolism and brain function may form part of a "microbiota-mouth-brain axis".

Keywords Alcohol drinkers, Cognitive impairment, Oral microbiota, Neuroinflammation, Nitrate

[†]Xiangxue Li, Zhaojun Ni and Weixiong Shi have contributed equally to the work.

*Correspondence:
Lei Su
sulei@cnilas.org
Yanxue Xue
yanxuexue@bjmu.edu.cn
Hongqiang Sun
sunhq@bjmu.edu.cn
[1] Peking University Sixth Hospital, Peking University Institute of Mental Health, NHC Key Laboratory of Mental Health (Peking University), National Clinical Research Center for Mental Disorders (Peking University Sixth Hospital), No.51 Huayuan North Road, Haidian District, Beijing 100191, China
[2] NHC Key Laboratory of Human Disease Comparative Medicine, Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (CAMS) & Comparative Medicine Center, Peking Union Medical College (PUMC), Beijing 100021, China
[3] Henan Collaborative Innovation Center of Prevention and Treatment of Mental Disorder, Henan Mental Hospital, The Second Affiliated Hospital of Xinxiang Medical University, Xinxiang, China
[4] Addiction Medicine Department, The Second People's Hospital of Guizhou Province, Guizhou, China
[5] National Institute On Drug Dependence and Beijing Key Laboratory of Drug Dependence, Peking University, Beijing 100191, China
[6] Department of Psychiatry, Pharmacology, Neuroscience, Immunology, Baylor College of Medicine, Houston, TX, USA
[7] Chinese Institute for Brain Research, Beijing, China

Introduction

Alcohol use disorder (AUD) occurs worldwide, with a 1.8% 12-month prevalence and a 4.4% lifetime prevalence in China [1]. The development of AUD mainly involves the modulation of biomarkers which are regulated by neurotransmitter or other neurochemical systems. The underlying factors for these cognitive dysfunctions as well as for negative affect and social withdrawal in alcoholics appears to involve various neurotransmitters and inflammation [2, 3], and neurochemical target-based therapy can be a better approach for the treatment of AUD [4]. Long-term alcohol consumption has been associated with liver disease [5], neuroinflammation [6], and elevated cancer risk [7]. Chronic alcohol-induced cognitive impairments include working memory, attention, and executive functions [8–12]. The underlying factors for these cognitive dysfunctions as well as for negative affect and social withdrawal in alcoholics appears to involve inflammation.

Dietary inorganic nitrate appears to be health-promoting through conversion of nitrate to nitrite and nitric oxide (NO) [13, 14]. The oral microbiota plays a critical role in nitrate conversion. Approximately 25% of dietary nitrate is actively taken up by the salivary glands and secreted into the oral cavity, where it is reduced to nitrite and NO by facultative anaerobic bacteria residing on the oral cavity, after which it is transported to various tissue [15, 16]. The NO released from nitrate can improve synaptic activity and reduce inflammation through inhibiting leukocyte recruitment to the site of inflammation such as the brain [17–19, 20]. Besides, inorganic nitrate consumption reduces aging-related cognitive impairment through nitrate metabolism by oral microbiota. Two distinct microbiome modules of co-occurring bacteria, that were sensitive to nitrate supplementation, showed stable relationships with cognitive (*Neisseria-Haemophilus*) and pro-inflammatory metabolism (*Prevotella-Veillonella).* *Prevotella-Veillonella* was diminished after nitrate supplementation [14, 21–23]. Thus, oral microbiota may modulate cognitive function through nitrite metabolism and an anti-inflammation response.

Gut microbiota composition also correlates with alcohol-induced neuropsychic behaviors [24, 25]. We previously showed that alcohol drinking led to diurnal flora disturbances in the oral microbiota and to associated cognitive impairment [26]. More broadly, gut microbiota existing in the mouth to the rectum have been associated with the pathogenesis of psychiatric diseases [27–31], including AUD and with cognitive impairment [32, 33]. Specifically, the oral microbiome composition has been associated with Alzheimer's disease [34, 35, 36–38], autism spectrum disorder [39], and post-traumatic stress disorder (PTSD) [40]. Among these, Alzheimer's disease has the most robust body of evidence, supported by both epidemiological and animal studies, demonstrating a strong association with alterations in oral microbiota. A particularly striking discovery is the identification of specific periodontal pathogens, such as *Porphyromonas gingivalis*, in the brains of individuals with Alzheimer's disease [41].

In the present study, we used 16S rRNA gene sequencing to test the impact of nitrate and alcohol on the oral microbiota. Our specific mouse tests included behavioral, serological, immunofluorescence tests and oral microbiota transplantation (OMT), in which Y-maze and novel-object recognition test were used to assess cognitive function. We then examined 63 alcohol drinkers with different levels of cognitive impairment to investigate whether lower concentrations of oral nitrate metabolism-related bacteria were associated with higher cognitive impairment and lower nitrate levels in plasma. We hypothesized that the oral microbiota was a requisite factor for nitrate to inhibit neuroinflammation and reduce alcohol-induced cognitive impairment.

Methods

Animal model

Specific pathogen-free (SPF) eight-week-old male C57BL/6 mice were bred at the National Institute on Drug Dependence, Peking University. The animals were housed under 12 h/12 h light/dark conditions with lights on from 8:00 AM to 8:00 PM and a controlled temperature of 21–22 °C and humidity of 55% ± 5%. Standard laboratory chow and water were provided ad libitum. Germ-free (GF) C57BL/6 mice (6–8 weeks) were bred and maintained in special plastic isolators (GemPharmatech, Nanjing, China). Animals were supplied with a 50-kGy irradiated sterile pelleted normal chow diet (Xietong Shengwu, Nanjing, China) and autoclaved tap water ab libitum. Bedding was replaced every 7 days. All germ-free mice were routinely screened for bacteria, viral, and fungus contamination.

For nitrate supplementation

The 45 SPF mice were divided into three dietary groups and fed with the liquid feed: (a) Healthy Control Group (HC group): control diet (fat comprising 10% of total calories, 72% of calories as carbohydrate); (b) Alcohol group (A Group): ethanol-containing diet (ingredient identical to the control diet but with ethanol added to account for 36% of total calories and the caloric equivalent of carbohydrate removed, provided by TROPHIC Animal Feed High-Tech Co., Ltd., Nantong, China); 36% ethanol-derived calories refers to the Lieber-DeCarli ad libitum diet [42], but we reduced the

fat composition and make it equal to the standard diet. (c) Alcohol and Nitrate Group (A + N group): ethanol and inorganic nitrate-containing diet (identical to the ethanol-containing diet but with sodium nitrate at 85 mg/kg/day; this amount is equivalent to a rich vegetable intake in humans [43]). Specifically, mice received fresh liquid feed in 50 ml feeders daily, between 7:00 PM and 8:00 PM. Body weight gain and food intake were assessed once a week. After 6 weeks of intervention, behavioral tests were conducted before euthanasia. Detailed are described under the behavioral test section.

For antiseptic mouthwash

Of the 30 SPF mice, the oral microbiota of 15 mice were depleted as the Chlorhexidine (CHX) group, while the others were categorized as the Control (Ctrl) group. To suppress the resident oral microflora, the CHX group was treated twice daily for 6 weeks with 0.3 ml of a commercial antibiotic mouthwash solution (0.2% wt/vol Chlorhexidine Gluconate/water; Longly, Wuhan, China). The Ctrl group received only a purified water solution as mouthwash [44–46]. All the mice were treated with ethanol and an inorganic nitrate-containing diet for 6 weeks. After 6 the week intervention, behavioral tests were conducted before euthanasia.

For oral microbiome transplantation

We recruited 3 additional male patients with a diagnosis of alcohol dependence according to DSM-IV. Participants provided baseline saliva (BL) samples and then received 14 days of dietary supplementation with concentrated NO3 −-rich beetroot juice (2 ×70 ml per day, organic beetroot juice each containing ~595 mg NO3 −, Beet it, James White Drinks, Ipswich, UK). They were instructed to consume one 70 ml beverage in the morning and another 70 ml in the afternoon each day, and on the last day, to consume their final beverage 1–3 h prior to post-intervention (PI) saliva collection. To ensure that recipient mice received similar inoculations, microbiota collected from donor patients of the same group were pooled. Bacteria were eluted and suspended in 1 ml sterile phosphate-buffered saline (PBS). Colony forming units (CFUs) were measured using a standard curve, as previously described [47]. Details are shown in Supplementary materials.

Out of 20 recipient GF mice, 10 received oral bacteria from the BL samples (rBL), and 10 received from the PI samples (rPI). Each germ-free mouse was orally inoculated with bacteria from donors twice with a one-day interval between inoculations, and a disposable injection syringe was used to pierce the gingival tissue [48]. After 14 days' intervention, behavioral tests were conducted before euthanasia.

Behavioral, immunological and bacterial RNA tests

The Y-maze and the novel-object recognition (NOR) tests were administered one or two days after the interventions. Before the mice were euthanized, oral swabs were taken of the entire oral cavity, including the tongue, maxilla, and teeth, and stored in 2-ml sterile tubes. The fasted mice were euthanized before collecting blood samples. The brains were removed immediately, and one half was fixed in 10% buffered formalin for histological analysis. The remaining half was frozen in liquid nitrogen and stored at −80 °C until further use. We used 16S rRNA sequencing for analyzing oral microbiota. The levels of IL-1β, IL-6, IL-10, TNF-α, and S100β were measured using an enzyme-linked immunosorbent assay (ELISA). Immunohistochemical analyses of ionized calcium-binding adapter molecule (Iba1) were conducted in sections of the hippocampus and medial prefrontal cortex (mPFC). See the Supplementary Materials for more details.

Human study participants

Sixty-three alcohol-dependent patients were recruited through advertisements. Patients were Han males with alcohol dependence according to the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) and the Mini International Neuropsychiatric Interview was adopted to rule out the subjects with other confounding disorders [49]. Patients were admitted to The Second People's Hospital of Guizhou Province and Anhui Mental Health Center and assessed for self-reported drinking conditions, including average drinks, age at first drink, drinking years, and withdrawal days. Patients were divided into two groups according to their cognitive function, measured by the Beijing version of the Montreal Cognitive Assessment (MoCA-BJ) [50]. In spite of the linguistic and cultural differences between the original English version and the Chinese version of the scale, the MoCA-BJ demonstrated an excellent sensitivity and a fair specificity under the recommended cut-off score of 26. The MoCA-BJ showed optimal sensitivity and specificity when the cut-off score was lowered to 22 [51]. So, we choose 22 as the cut-off score to divide alcohol drinkers to two groups. The MoCA sores of the alcohol drinkers in group AD-LC (Alcohol drinkers with lower cognitive function, N = 27) had cognition less than 22, while those in the group AD-HC of alcohol drinkers (Alcohol drinkers with higher cognitive function, N = 36) had better cognition with scores greater than or equal to 23.

Bioinformatics analysis of bacterial flora

Alpha and beta diversity were estimated using MOTHUR (v1.31.2) [52] and QIIME (v1.8.0) [53] at the OTU level, respectively. Sample clusters were conducted by QIIME (v1.8.0) based on UPGMA. Kyoto Encyclopedia of Genes and Genomes (KEGG) functions were predicted using PICRUSt2 (v2.3.0-b) software [54]. Statistically significant KEGG pathways for each group were additionally identified using LEfSe. Bar plots of classification levels were plotted with R package v3.4.1 and R package gplots. Principal component (PCA) analysis in OTUs was plotted with R package ade4. Partial least-squares discrimination analysis was performed by R package mixOmics. Principal Coordinate Analysis (PoCA) was performed using QIIME (v1.8.0) [55]. Linear discriminant analysis effect size (LEfSe) cluster or linear discriminant analysis (LDA) analysis was conducted by LEfSe. Significant Species or functions were determined by R (v3.4.1) based on Wilcox-test or Kruskal-Test.

Statistical analysis

The statistical analysis was performed using SPSS 20.0 software (SPSS, Chicago, IL, USA). Data were assessed for a normal distribution and expressed as the mean ± standard error of the mean. Differences between the two groups were assessed using the two-tailed, unpaired Student's t-test or the Mann–Whitney U test. Other continuous variables were expressed as the median (Q25, Q75) and analyzed by a nonparametric test. The discontinuous variables were analyzed by the chi-square test. For the comparisons of more than two groups, a one-way analysis of variance (ANOVA) was used, followed by the post hoc Tukey–Kramer test for multiple comparisons. A p value of < 0.05 was considered statistically significant.

Results

Dietary nitrate protects against alcohol-induced cognitive impairment and restores alcohol-induced oral microbiota changes in SPF mice

To determine the causal link between nitrate metabolism and alcohol-induced cognitive impairment, we examined whether nitrate supplementation prevents alcohol-induced cognitive impairment in mice. We divided SPF mice into three groups: healthy control diet (HC), alcohol diet (A), and alcohol with extra inorganic nitrate supplements (A + N) (Fig. 1A). We found A group gained less weight after six weeks than controls ($F_{2,39} = 7.025$, $p < 0.01$; Post hoc A vs. HC $p < 0.01$). The mean weight of the A + N group mice showed no significant difference from the other two groups except in day 42 (Fig. 1B). Food intake was showed in Supplementary Fig. 1 A.

We tested the effects of nitrate supplementation on cognitive function in mice using the NOR and Y-maze behavior tests. Cognitive performance was severely disrupted in the A group, and these effects were less severe in A + N group. Spontaneous alteration in the Y-maze test increased by 17.43% in the A + N group compared with the A group ($F_{2,39} = 10.042$, $p < 0.001$; post hoc: A vs. HC $p < 0.001$; A vs. A + N $p < 0.05$) (Fig. 1C). Similarly, the NOR test showed a rise in the percentage of novelty preference ($F_{2,39} = 3.590$, $p < 0.05$; post hoc: A vs. HC $p = 0.017$; A vs. A + N $p > 0.05$) in the HC group mice compared with the A group, A + N group showed no significant difference with the two group (Fig. 1D). Thus, nitrate can weaken alcohol's impairment of cognitive function, body weight, and food consumption.

To explore the possible link of oral bacteria in cognitive improvement by nitrate, we performed 16S rRNA gene sequencing on saliva samples in the HC, A, and A + N groups. Nitrate prevent a drastic shift in the oral microbiota composition in mice with alcohol administration, making their oral microbiota

(See figure on next page.)

Fig. 1 Dietary nitrate benefits cognition and restores the oral microbiota changes caused by alcohol. **A** Schematic diagram showing the study design for alcohol and nitrate supplementation. **B** The changes in body weight during six weeks among the three groups: group A had the lowest weight gain, while group HC had the highest body weight gain, with significant differences compared with group A. The body weight gain in group A + N was not statistically significant different from the other two groups except on day 42 (n = 13–14). **C** and **D** Behavioral tests showed that novelty preference in the novel-object recognition (NOR) test and spontaneous alternation in the Y-maze test were significantly decreased in group A mice compared with controls, and nitrate intake could improve cognitive function of mice with alcohol in behavioral tests (n = 13–14). **E** In group A, the Chao index of the oral microbiome was significantly higher compared with that in group HC, but nitrate consumption could lessen the difference (n = 13–14). **F** The weighted UniFrac distance varied among the three groups, demonstrating significant differences in the oral microbiota of the three groups with different kinds of food intakes (n = 13–14). **G** The operational taxonomic unit (OTU) Core-Pan showed that samples in group A had much more extra OTUs than the other two groups (n = 13–14). **H** and **I** Each vertical bar represents the relative abundance of the oral sample in different groups. The composition of the microbiota was substantially different among the three groups (n = 13–14). **H** at the phylum level, **I** at the genus level. **J**–**P** Genus-level differences in the relative abundance of Streptococcus, Corynebacterium, Escherichia, Lactobacillus, Pasteurella, Megasphaera and Megamonas (n = 13–14). **Q** Among level-3 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, differences in the lipopolysaccharide biosynthesis modules (n = 13–14). The data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

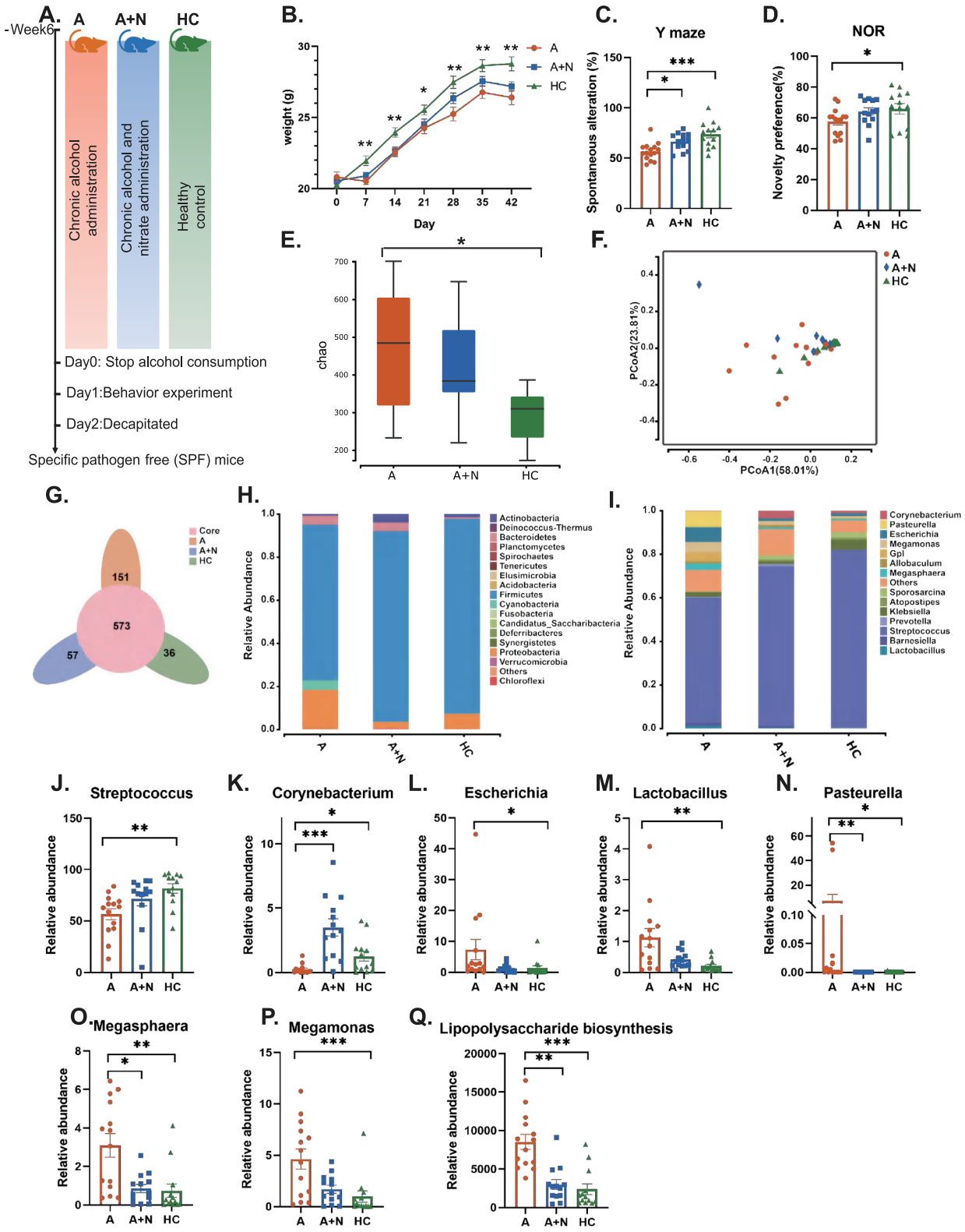

Fig. 1 (See legend on previous page.)

indistinguishable from that of the HC group. The Chao ($F_{2,38}$ = 8.474, $p < 0.05$) index of the microbiota substantially increased after alcohol consumption, while the Chao index of the oral flora was reduced almost to the level of that in the HC group after nitrate administration (Fig. 1E). We used the UniFrac distance metric to assess the effects of nitrate and alcohol on between-individual variation in microbiota community composition and found the oral microbiota from the group A mice differed from the other two groups based on PCoA, suggesting evident differences in the composition of oral bacteria between alcohol drinkers and alcohol drinkers with nitrate supplementation (similar to healthy control mice, $p < 0.01$, PERMANOVA; Fig. 1F).

The effect of alcohol and nitrate on altering the oral microbiota was also evident in measurements of the abundance of specific oral microbiota taxa. The operational taxonomic unit (OTU) Core-Pan showed that all groups had 573 OUT tags in common, while samples in group A had 151 extra OTU tags. In contrast, the HC and A + N groups had 36 and 57 distinct OTUs, respectively (Fig. 1G). The community composition changed considerably after alcohol administration at the phylum level but was preserved when simultaneously supplementing nitrate, especially among Firmicutes, Cyanobacteria, Proteobacteria, and Actinobacteria (Fig. 1H). At the genus level, the same situation obtained (Fig. 1I) (Supplementary Fig. 2). Distinct composition was observed in group A by the growth of bacteria, included more *Escherichia* (χ^2 = 7.252, $p < 0.05$), *Lactobacillus*, (χ^2 = 11.980, $p < 0.01$), *Pasteurella* (χ^2 = 34.862, $p < 0.01$), *Megasphaera* (χ^2 = 13.230, $p = 0.001$) and *Megamonas* (χ^2 = 12.370, $p < 0.01$) (Fig. 1L–P). Meanwhile, the abundance of the nitrate metabolism-related microbiota, *Streptococcus*, decreased in group A (χ^2 = 12.440 $p < 0.01$) (Fig. 1J) while the abundance of *Corynebacterium* increased in group A + N (χ^2 = 18.840, $p < 0.001$) (Fig. 1K).

We also found that immune-related Kyoto Encyclopedia of Genes and Genomes (KEGG) functional modules were reinforced in group A, which included lipopolysaccharide biosynthesis (χ^2 = 20.090, $p < 0.000$) (Fig. 1Q). These findings suggest that nitrate supplements partly restore the oral microbiota impaired by alcohol and inhibit the growth of some potential pathogenic bacteria. Nitrate metabolism-related bacteria also increased because of additional nitrate.

Nitrate prevents alcohol-induced inflammatory activation in SPF mice

Previous studies found that chronic alcohol consumption induced a rapid increase in the activation of the neuroinflammatory response and affected the function of many brain regions [56], including the medial prefrontal cortex (mPFC) and hippocampus. The hippocampus is a critical brain region for modulating memory, navigation, and cognition [57]. The mPFC receives innervation from the brainstem and hippocampus and plays a fundamental role in attention and memory [58].

Therefore, we hypothesized that nitrate would inhibit the activation of inflammatory responds, which reflected as the collection of microglia in the brain [59]. In group A, we observed an increase in the number of Iba1-positive cells in various areas of the hippocampus and mPFC, including hippocampal CA1 ($F_{2,36}$ = 15.090, $p < 0.001$), hippocampal CA3 ($F_{2,36}$ = 33.980, $p < 0.001$), dentate gyrus (DG, $F_{2,36}$ = 20.110, $p < 0.001$), dorso-medial prefrontal cortex (dmPFC, $F_{2,36}$ = 113.800, $p < 0.001$), and ventro-medial prefrontal cortex (vmPFC, $F_{2,36}$ = 11.160, $p < 0.001$) (Fig. 2A–F).

Activated microglia release proinflammatory cytokines, which lead to the compromise of the blood–brain barrier (BBB) [60, 61]. We found that alcohol consumption led to an increase in proinflammatory cytokines IL-1β ($F_{2,19}$ = 27.950, $p < 0.001$), IL-6 ($F_{2,19}$ = 7.982, $p < 0.01$) and TNF-α ($F_{2,19}$ = 2.698, $p = 0.094$), and a decrease in the anti-inflammatory cytokine IL-10 ($F_{2,19}$ = 10.674, $p < 0.001$). And this proinflammatory status were partially preserved by nitrate supplementation in group A + N (Fig. 2G–J). S100β is a biochemical marker of brain injury [62] and also significantly increased ($F_{2,19}$ = 4.788, $p < 0.05$) in group A compared with the HC groups (Fig. 2K).

There were significantly higher proinflammatory cytokine levels in serum, including IL-1β ($F_{2,19}$ = 13.461, $p < 0.001$) and IL-6 ($F_{2,19}$ = 5.670, $p < 0.05$) in group A than that in the other two groups. Proinflammatory cytokine TNF-α ($F_{2,19}$ = 2.522, $p = 0.108$) and anti-inflammatory cytokine levels IL-10 ($F_{2,19}$ = 0.823, $p = 0.455$) did not change significantly (Supplementary Fig. 3 A–D).

These findings suggest chronic alcohol consumption causes increased inflammatory responses in the central neuron system and the peripheral circulation, while nitrate supplementation may inhibit these immune responses.

Elimination of the oral microbiota weakens nitrate's protective effects on cognition and inflammation in alcohol-exposed mice

Next, to explore whether the oral-residing microflora contributed to cognitive function and inflammatory suppression induced by nitrate in alcohol consuming mice, we assessed whether the absence of the oral microbiota influences inflammation and cognitive function after nitrate and alcohol supplementation for 6 weeks by comparing Ctrl mice with CHX mice. The CHX group received Chlorhexidine treatment twice

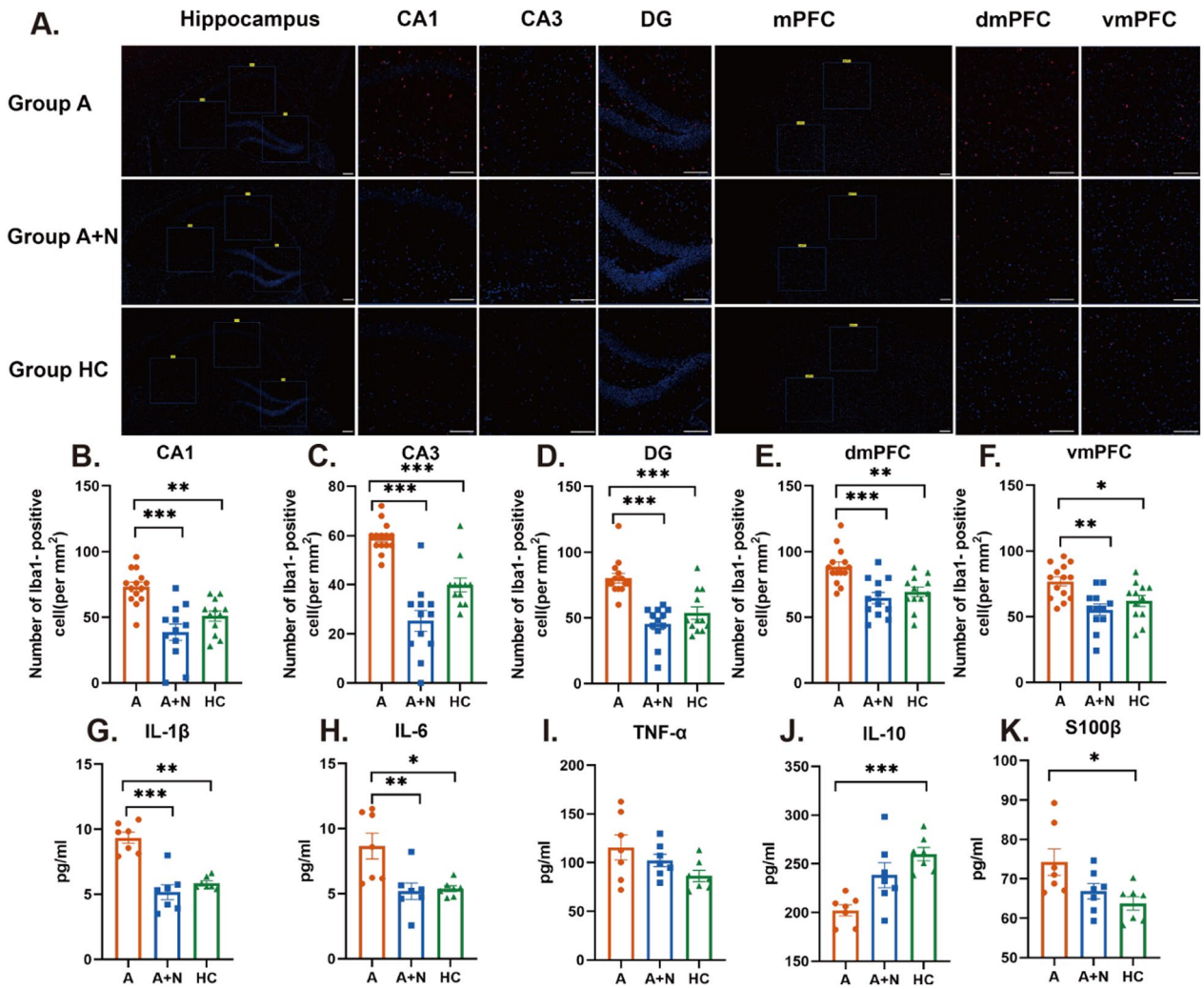

Fig. 2 Nitrate prevented inflammatory activation induced by alcohol consumption. **A** Representative images of Iba1 immunofluorescence in the CA1, CA3 and DG (hippocampus) and dmPFC and vmPFC (medial prefrontal cortex, mPFC). Scale bar = 100 μm. Higher definition picture was shown in Supplementary *Fig. 6*. **B**–**F** Quantification of the number of Iba1-positive microglia in the CA1, CA3, DG, vmPFC and dmPFC. Group A exhibited a higher number of Iba1-positive cells compared with the other two groups (n = 13–14). **G**–**J** Higher levels of proinflammatory cytokines IL-1β and IL-6 and lower levels of anti-inflammatory cytokine IL-10 were found in brain tissue in group A, compared with the other two groups (n = 7 per group). **K** Higher levels of S100β in the serum of group A than that in the other two groups (n = 7 per group). The data are expressed as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001

daily for 6 weeks, while the Ctrl group was treated with saline (Fig. 3A). It was observed that the weight of the Ctrl mice significantly increased starting from the 14 th day (Fig. 3B). Food intake was showed in Supplementary Fig. 1B. The venn diagram showed that samples in group CHX had 158 extra OTU tags while the Ctrl groups had 673 extra OUTs (Supplementary Fig. 4A). Meanwhile, the composition of the oral microbiota in two groups showed a significant difference (Supplementary Fig. 4D).

The effects of oral microbiota depletion on cognitive function were tested in mice using the NOR and Y-maze behavior tests. Spontaneous alteration in the Y-maze test decreased with significant difference in the CHX group than the Ctrl group ($t = 3.533$, $p < 0.01$) (Fig. 3C). However, the NOR test showed no significant difference in the percentage of novelty preference ($p > 0.05$) (Fig. 3D).

Subsequently, we investigated whether the elimination of oral microbiota would attenuate the anti-inflammatory effects of nitrate. A heightened inflammatory response was observed in the hippocampus and mPFC of CHX mice compared with Ctrl mice. There was an increase in the number of Iba1-positive cells in the CHX group compared with the Ctrl group, including hippocampal

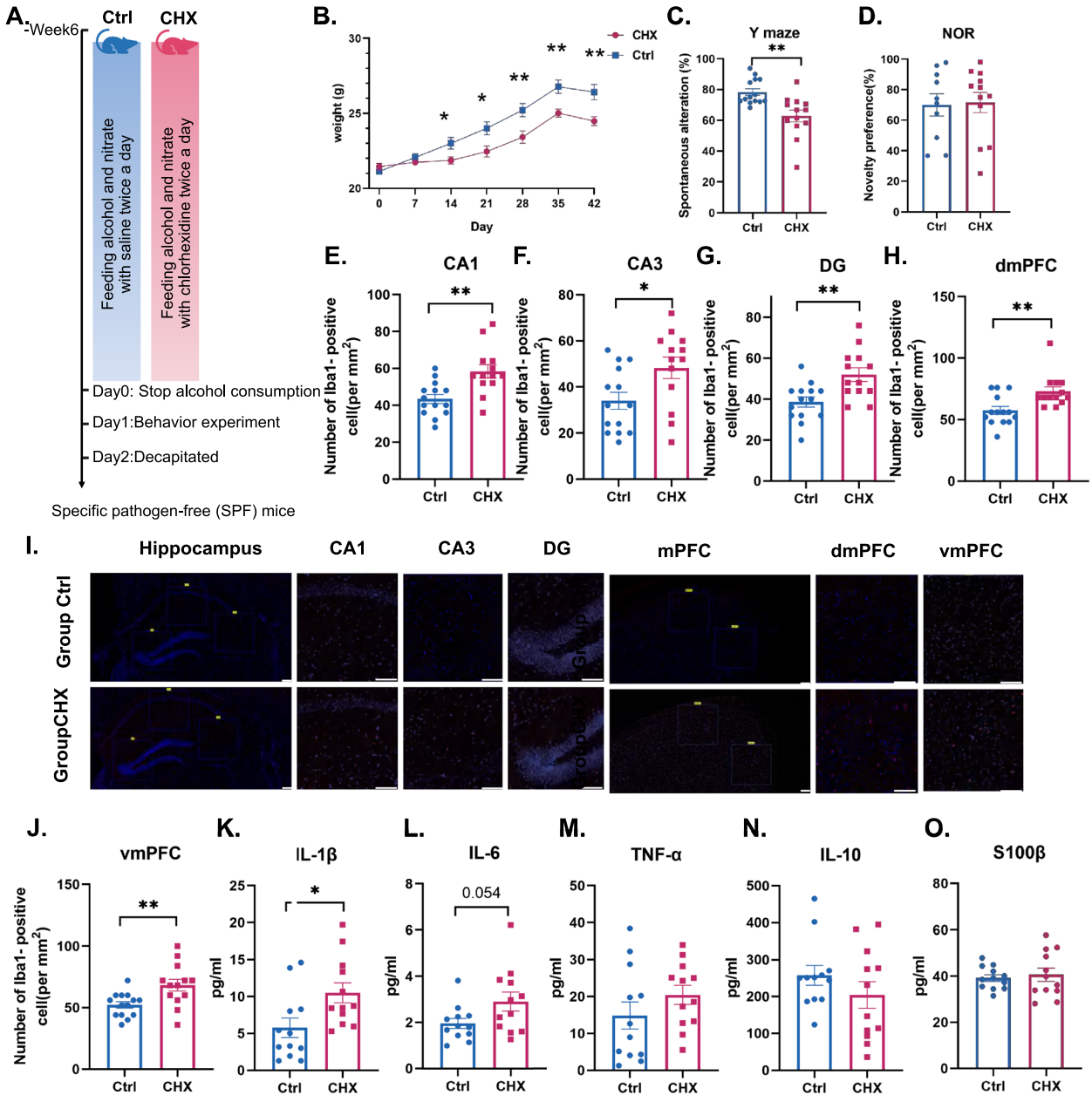

Fig. 3 Elimination of oral microbiota weakens nitrate's protective effects on cognition and inflammation in alcohol-exposed mice. **A** Schematic diagram showing the study design for oral microbiota removal. **B** The changes in body weight during six weeks (n = 13–14). **C** and **D** Behavioral tests showed that spontaneous alternation in the Y-maze test were significantly decreased in Chlorhexidine (CHX) group compared with Control (Ctrl) group, while novelty preference in the NOR test decreased with no statistic difference (n = 13–14). **E**–**G** Quantification of the number of Iba1-positive microglia in the CA1, CA3 and DG (n = 13–14). **H** and **J** Quantification of the number of Iba1-positive microglia in the vmPFC and dmPFC (n = 13–14). **I** Representative images of Iba1 immunofluorescence in the CA1, CA3, DG (hippocampus) and dmPFC, vmPFC (medial prefrontal cortex, mPFC), Scale bar = 100 μm. Higher definition picture was shown in Supplementary *Fig. 7*. **K**–**N** Significant increases in the proinflammatory cytokines IL-1β and IL-6 were found in brain tissue in the CHX group, compared with group Ctrl. TNF-α and IL-10 in brain tissue showed no significant difference between the two groups (n = 12 per group). **O** S100β in the serum showed no significant difference between the two groups (n = 12 per group). The data are expressed as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001

CA1 ($t = 3.514$, $p < 0.01$), hippocampal CA3 ($t = 2.426$, $p < 0.05$), dentate gyrus (DG, $t = 3.274$, $p < 0.01$), dorso-medial prefrontal cortex (dmPFC, $t = 3.121$, $p < 0.01$), and ventro-medial prefrontal cortex (vmPFC, $t = 2.993$, $p < 0.01$) (Fig. 3E–J). The absence of oral microbiota was found to promote pro-inflammatory phenotypes in the brain tissue of the CHX group, evidenced by an increase in pro-inflammatory cytokines IL-1β ($t = 6.284$, $p < 0.05$), IL-6 ($t = 4.163$, $p = 0.054$) and TNF-α ($p > 0.05$) and alongside a decrease in the anti-inflammatory cytokine IL-10 ($p > 0.05$) (Fig. 3K–N). An increase in S100β was observed in the CHX group, though not reaching statistical significance ($p > 0.05$) (Fig. 3O). In peripheral circulation, inflammatory markers exhibited a similar trend to that observed in brain tissue, albeit without statistical significance (Supplementary Fig. 3E–H).

In summary, these results indicate that oral microbiota plays an important role in the nitrate-induced cognitive protection and inflammatory suppression.

Transplantation of the oral microbiota from nitrate-supplemented donors improves cognitive function and reduces neuroinflammation in GF mice

To explore the role of oral microbiota in mediating cognitive improvement and mitigating neuroinflammation, oral microbiota samples from alcohol drinkers, collected before and after 14 days of nitrate supplementation, were randomly selected for transplantation into GF mice (Fig. 4A). Microbiota inocula, derived from pooled baseline (BL) and post-intervention (PI) oral samples (labeled rBL and rPI, respectively), were prepared from three randomly selected alcohol drinkers, chosen without prior knowledge of their microbial diversity profiles. Following 14 days of supplementation, significant changes occurred in the oral microbiota composition of the subjects, notably increases in *Neisseria* and *Haemophilus,* alongside decreases in *Veillonella* (Fig. 4B).

Two weeks after OMT, we examined the composition of the oral microbiota in two groups and found increases in the abundance of *Haemophilus* ($Z = 2.531$, $p = 0.010$) (Fig. 4D). the abundance of *Neisseria* showed no significant difference but an increase tendency while the abundance of *Veillonella* and *Prevotella* showed an decrease tendency ($p > 0.05$) (Fig. 4E, Supplementary Fig. 4B, C). The composition of the oral sample in the rBL and rPI groups after OMT changed a lot at genus level (Supplementary Fig. 4E). No statistically significant difference in body weight was observed between the two groups (Fig. 4C); in the meantime, the rPI mice demonstrated improved performance in cognitive behavior tests after OMT. Specifically, a significant increase in spontaneous alteration was observed in the Y-maze test ($t = 2.200$, $p < 0.05$), whereas the changes in novelty preference in the NOR test was not statistically significant (Fig. 4F–G).

Subsequently, we examined the inflammatory responses in the rBL and rPI groups after OMT. A reduction in inflammatory responses was observed in the GF mice transplanted with PI oral microbiota in the hippocampus and mPFC, comparing with the rBL group. There was a decrease in the number of Iba1-positive cells in rPI group (Fig. 4H), including hippocampal CA1 ($t = 2.613$, $p < 0.05$), hippocampal CA3 ($p > 0.05$), dentate gyrus (DG, $t = 3.160$, $p < 0.01$), dorso-medial prefrontal cortex (dmPFC, t 2.444, $p < 0.05$), and ventro-medial prefrontal cortex (vmPFC, $t = 2.118$, $p < 0.05$) (Fig. 4I–M). Transplantation with the PI oral microbiota resulted in suppression of pro-inflammatory phenotypes in the rPI group's brain tissue, marked by an increase in pro-inflammatory cytokines IL-1β ($t = 2.322$, $p < 0.05$), IL-6 ($t = 2.674$, $p < 0.05$) and TNF-α ($t = 2.001$, $p = 0.062$). (Fig. 4N–P). IL-10 and S100β showed no significant difference between the two groups ($p > 0.05$) (Fig. 4Q–R). In peripheral circulation, the inflammatory factor showed the same tendency as the brain tissue but with no statistically significant difference (Supplementary Fig. 3I–L).

Collectively, these findings demonstrate that transplantation of human oral microbiota following

(See figure on next page.)

Fig. 4 Transplantation of nitrate-supplemented oral microbiota improves cognitive function. **A** Schematic diagram showing the study design for OMT. **B** The composition of the oral microbiota of two groups at the genus level (n = 9–10). **C** The changes in body weight during two weeks (n = 9–10). **D** and **E** Differences in the relative abundance of Neisseria and Haemophilus (n = 9–10). **F** and **G** Behavioral tests showed that spontaneous alternation in the Y-maze test were significantly higher in the rPI group compared with the rBL group, while novelty preference in the NOR test did not differ with statistically significant difference (n = 9–10). **H** Representative images of Iba1 immunofluorescence in the CA1, CA3, DG (hippocampus) and dmPFC and vmPFC (mPFC). Scale bar = 100 μm. Higher definition picture was shown in Supplementary *Fig. 8.* **I**–**M** Quantification of the number of Iba1-positive microglia in the CA1, CA3, DG, vmPFC and dmPFC. The rPI group exhibited significant decreases in the number of Iba1-positive cells compared with the rBL group (n = 9–10). **N**–**Q** Significant decreases in the proinflammatory cytokines IL-1β and IL-6 were found in brain tissue in group rPI compared with group rBL. TNF-α and IL-10 in brain tissue showed no significant differences between the two groups (n = 9–10). **R** S100β in the serum showed no significant difference between the two groups (n = 9–10). The data are expressed as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001

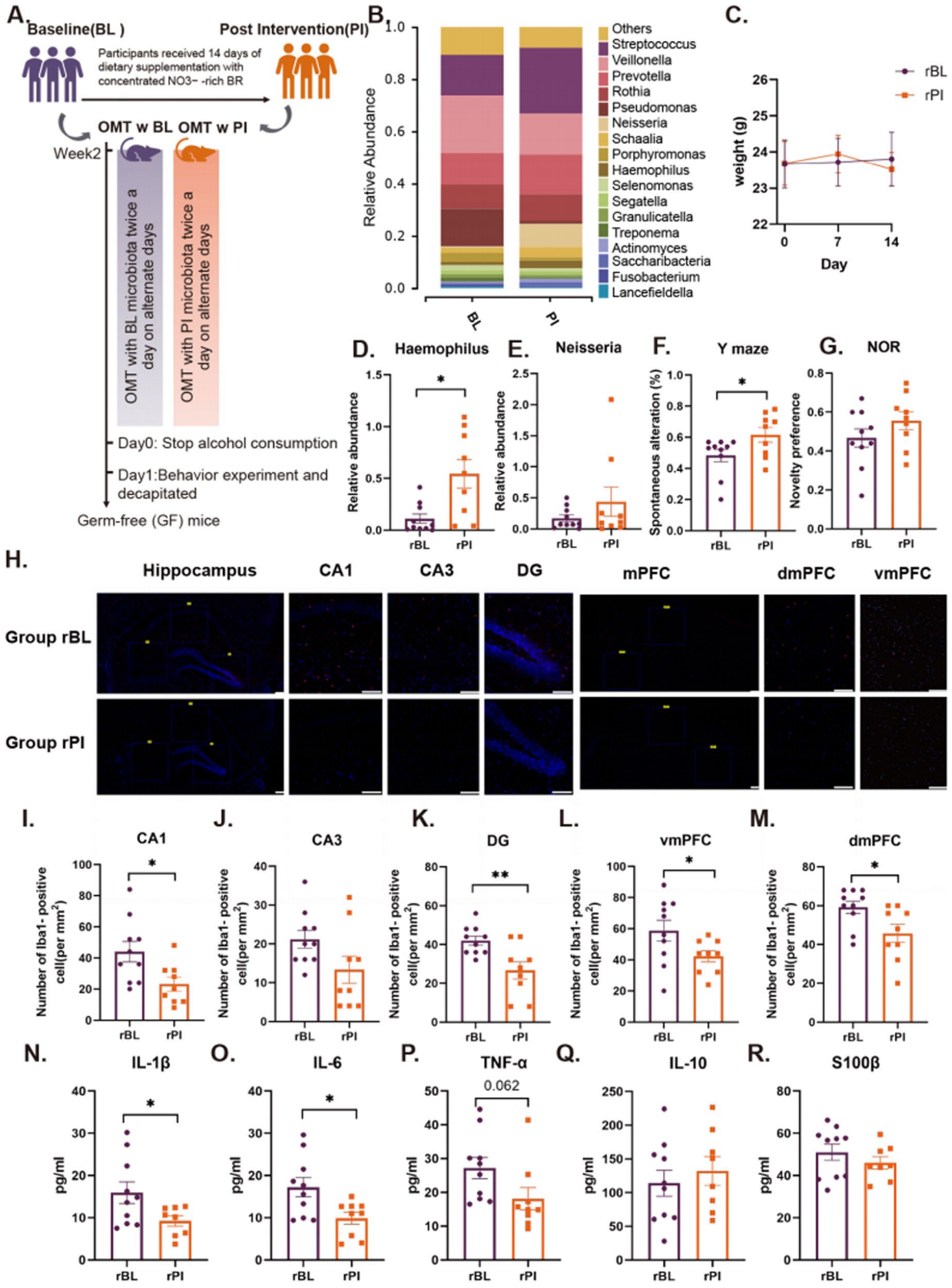

Fig. 4 (See legend on previous page.)

nitrate supplementation enhances cognitive function and reduces neuroinflammation in OMT mice compared to those receiving OMT from the subjects without nitrate supplementation.

Greater cognitive impairment in alcohol drinkers is associated with reductions in nitrate metabolism-related bacteria

To clinically examine the relationship between oral microbiota, nitrate consumption and alcohol-induced cognitive impairment, we divided the alcohol drinkers by the total MoCA-BJ score into low AD-LC and high AD-HC impairment groups. Several MoCA cognitive areas showed the largest differences: attention ($\eta^2 = 0.300$, $Z = 4.651$, $p < 0.001$), language ($\eta^2 = 0.376$, $Z = 4.830$, $p < 0.001$), delayed recall ($\eta^2 = 0.536$, $Z = 5.714$, $p < 0.001$) and execution ($\eta^2 = 0.390$, $Z = 4.895$, $p < 0.001$) (Fig. 5A). The two alcohol drinker groups did not differ in demographics or alcohol use characteristics except for education years and annual household income as shown in Table 1. Additionally, the plasma nitrate concentration was lower in patients with lower MoCA scores (AD-LC $= 1.939 \pm 0.163$, AD-HC $= 3.894 \pm 0.779$; $t = 2.571$, $p < 0.05$), suggesting that the cognitively impaired group (AD-LC) was less able to convert nitrite to nitrate and NO, which is our hypothesized role for the specific oral bacterial deficiency associated with alcohol-induced cognitive impairment.

Although the AD-LC and AD-HC groups showed no significant difference in the Chao index, the groups

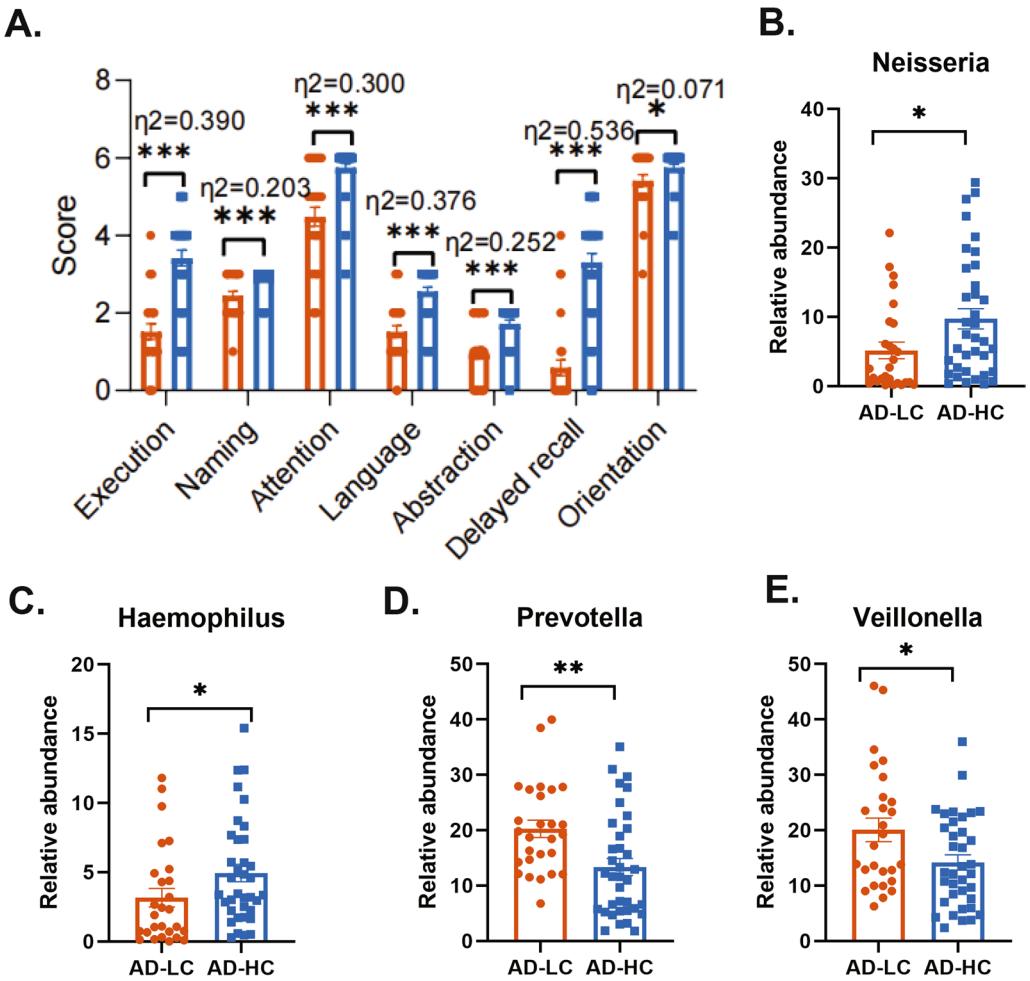

Fig. 5 Higher cognitive impairment in alcohol drinkers is associated with reductions in nitrate metabolism-related bacteria and increased inflammatory responses. **A** As expected from the selection of the two human subject groups, differences in the specific Montreal Cognitive Assessment (MoCA) items: The scores of attention, language, memory and execution were significantly lower in the alcohol drinkers with lower cognitive function (AD-LC) group than in the alcohol drinkers with higher cognitive function (AD-HC) group. **B**–**E** Genus-level differences in the relative abundance of Neisseria, Haemophilus, Prevotella and Veillonella between the two groups. $p < 0.05$ and permutation $p < 0.05$ for all data selected. The data are expressed as the mean ± SEM ($n = 63$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 1 General demographic information

Item	AD-LC (n = 27)	AD-HC (n = 36)	Z/χ/t	p value
Age(year)	44 (40,48)	37 (32,48.75)	366.5	0.097
Education years	8 (6,9)	10.5 (9,15)	780.00	0.000***
Marriage status	/	/	1.18	0.277
Married	22 (81.48%)	25 (69.44%)	/	/
Single/Divorced/Widowed	5(18.52%)	11 (30.55%)	/	/
Income (yuan/year)	50000 (15000, 150000)	100,000 (52500, 200000)	653.00	0.019*
BMI (kg/m^2)	21.56 ± 2.92	23.14 ± 3.71	− 1.83	0.072
Withdrawal days	8.00 (3.00, 15.00)	7.50 (3.00, 18.50)	490.00	0.956
Drinking years	10.00 (5.00, 15.00)	6.50 (3.25, 17.75)	438.50	0.507
Age at first drink	17.00 ± 2.67	17.19 ± 3.38	− 0.25	0.806
Average drinks in a day (standard drinks)	12.00 (7.00, 21.00)	15.00 (9.00, 25.68)	631.5	0.060

AD alcohol dependence, *BMI* body mass index

*$p < 0.05$, ***$p < 0.001$

differed in the weighted UniFrac distances, which assesses β-diversity in microbiota ($p < 0.01$, PERMANOVA). When we then examined specific differences at the genus level, the AD-LC group showed markedly lower *Neisseria* ($Z = 2.458$, $p < 0.05$) and *Haemophilus* ($Z = 2.375$, $p < 0.05$) and greater *Prevotella* ($Z = -3.125$, $p < 0.01$) and *Veillonella* genuses ($Z = -2.292$, $p < 0.05$) (Fig. 5B–E).

Discussion

Alcohol and chronic alcohol drinking affects oral mucosa, salivary glands, and saliva and impairs cognitive function [63]. In addition, alcohol consumption may influence the amount, composition, and diurnal rhythm of oral microbiota [26, 64], which is consistent with the oral microbiome's association with other psychiatric disorders [36, 39, 40]. Interestingly, inorganic nitrate consumption has improved cognitive impairment caused by aging through the activity of nitrate metabolism-related oral microbiota, including *Neisseria* and *Haemophilus*, while *Prevotella*, and *Veillonella* has been associated with pro-inflammatory metabolism was diminished after nitrate supplementation [14, 21–23].

The mechanisms of nitrate action are highly complex, and its direct effects on neurovascular function could indeed represent an alternative pathway influencing cognitive outcomes. Previous studies have demonstrated that NO exerts beneficial effects on neurovascular function, primarily by inducing the dilation of cerebral vessels and enhancing cerebral blood flow then influence the cognitive function [65]. The present study demonstrated the specific contributions of oral microbiota to alcohol-induced cognitive impairment and identified a potential mechanism involving nitrite conversion to NO and its associated inhibition of neuroinflammation. In mice, nitrate supplementation protected against alcohol-induced cognitive impairment, potentially by inhibiting neuroinflammation in the hippocampal and PFC regions. Removal of the oral microbiota diminished the beneficial effects of nitrate supplementation. Then, transplantation into germ-free mice of oral microbiota from AUD subjects given the oral nitrate intervention improved cognitive function and ameliorated neuroinflammation induced by alcohol in these germ-free mice. These studies underscored the close association between the oral microbiota and brain function within a"microbiota-mouth-brain axis". We also analyzed clinical data and found chronic AUD was associated with the alteration of specific oral microorganisms involved in nitrate metabolism. We tested the concentration of nitrate in serum and found significantly lower levels in the group with more cognitive impairment. These findings suggest that a nitrate-rich diet in conjunction with the appropriate oral microflora might play a critical role in protecting against alcohol-induced cognitive impairment.

Alcohol intoxication leads to CNS inflammation and neurodegeneration [66], characterized by increased expression of proinflammatory cytokines (e.g., TNFα, IL-1β, and CCL2) and microglial activation. Oral nitrate conversion to nitrite and NO generates a potent anti-inflammatory mediator [15, 20]. In the present study, we found that mice given chronic alcohol showed worse cognitive behavior performance and more pronounced immune responses than control mice. Alcohol-induced inflammatory responses in the brain included an increase in the proinflammatory cytokines IL-1β, IL-6, and TNF-α and a decrease in the anti-inflammatory cytokine IL-10 [67, 68]. We also observed an increase in microglial activation reflecting chronic neuroinflammation in various areas of the hippocampus and medial PFC, which

previous studies found was related to dysfunction in these brain areas [57, 69–71].

Previous studies with samples of rat tongue or human saliva have implicated *Micrococcus, Corynebacterium, Propionibacterium, Neisseria,* and *Actinomyces* as critical to oral nitrate reduction [72, 73]. Like our human study, our mouse experiment showed increases in the abundance of *Corynebacterium* after nitrate administration, but no change in *Neisseria*. Oral microbiota depletion in mice weakened but did not eliminate the of nitrate effect on cognitive function. This discrepancy probably reflects basal activity of nitrate reduction-related enzymes such as xanthine oxidoreductase, which are active in rodents, but not in humans [74, 75]. Nevertheless, the oral microbiota in mice still plays a vital role in the metabolism of dietary nitrate, as demonstrated by decreased plasma nitrite levels in mice lacking oral bacteria [46].

Bacterial effects are critical for immune system development in infants [76] and for maintaining immune cell functions in the adult intestine [77]. Because of the close anatomical position, oral pathogens can enter the brain, potentially affecting memory and causing dementia [78]. Bacteria entering through the BBB into the brain tissue promotes glial activation, neuroinflammation and neuron degeneration [79–81]. For example, oral bacterial pathogens are associated with the risk of Alzheimer's disease [82, 83]. High-throughput sequencing was employed to analyze the bacterial composition of the oral microbiome in snap-frozen brain tissues from individuals with Alzheimer's disease and non-Alzheimer's disease controls. The results revealed that *Prevotella* and *Veillonella* were uniquely detected in the Alzheimer's disease group, suggesting a potential association between these bacterial and the brain disease [41]. Other studies have also suggested that *Prevotella* and *Veillonella* are associated with pro-inflammatory responses [84]. Similarity, we found lower levels of *Prevotella* and *Veillonella* in AUD patients with better cognitive function.

Our KEGG analysis revealed that inflammation-related pathways, such as NOD-like receptor signaling and the lipopolysaccharide (LPS) biosynthesis pathway were upregulated after alcohol exposure but inhibited after nitrate supplementation. LPS is an abundant molecule in the outer membrane of bacteria that is associated with chronic infection [85]. NOD-like receptor signaling activates the NF-κB and MAPK pathways, producing proinflammatory cytokines and chemokines [86]. By transplanting the oral microbiota, GF mice showed different degrees of cognitive impairment and inflammatory responses. The enhanced inflammatory response related to bacterial and functional pathways suggests that inflammation may play a critical role in alcohol-induced cognitive impairment, and nitrate supplementation may attenuate inflammatory immune responses.

To our knowledge, we are the first to find the beneficial effects of nitrate on alcohol induced cognitive injury and elucidate the oral microbiota's role in the mechanism. However, the oral cavity is regarded as an endogenous reservoir for gut microbiota, as oral microbes translocate and colonize the intestine [87]. Furthermore, administration of chlorhexidine as well as oral microbiota transplantation has an impact on gut microbiota, and previous studies have shown that gut microbiota also can impact memory function and inflammation parameters [88]. Thus, we are the first study to publish a link between oral microbiota, inflammatory responses, and cognitive decline by OMT, we have modestly extended the role of the microbiota-gut-brain axis to explicitly include the oral cavity. However, we have collected the gut microbiota and will further analyze those data to clarify this gut-brain relationship.

Finally, while we also have clinical support in a 63 patients cross-section study. The clinical conclusions draw on previous studies showing that oral *Neisseria* and *Haemophilus* concentrations importantly contribute to converting nitrite to nitrate and that nitrate can reduce cognitive impairment that toxic agents like alcohol can induce [21, 73]. Specific support for our conclusions include finding that nitrite levels were high in cognitively impaired AUD patients suggesting that their ingestion of inorganic nitrites were not being converted efficiently into nitrate and NO. Two associations among the low impairment AUD patients further supported nitrate's protection from alcohol's induced cognitive impairment. First, their low levels of plasma nitrites were presumably due to conversion to nitrates and NO. Second, their markedly greater oral concentrations of oral *Neisseria* and *Haemophilus* provided a mechanism for this biochemical conversion of nitrite. These two associations do not prove causation but suggest testable clinical hypotheses for proof of concept studies such as a randomized double-blind placebo-controlled clinical trial to test the effect of nitrate-rich juice on cognitive function in patients with AUD. We also conducted such a study along with measures of the oral and gut microbiome.

These findings highlight the therapeutic potential of nitrate supplementation and oral microbiota probiotics as clinically relevant interventions for alcohol-induced cognitive impairment. While nitrate-rich beetroot juice has been widely used as a dietary supplement,

the application of oral microbiota probiotics in brain-related disorders is particularly intriguing, given the established association between oral bacteria and neural inflammation as well as brain function. To further elucidate the underlying mechanisms, future studies should employ metagenomic and metabolomic analyses to investigate the functional roles of specific microbial communities and bacterial species. Collectively, this series of studies not only proposes a novel strategy for addressing the neuropathology of AUD but also offers potential therapeutic avenues for other neurodegenerative diseases, which share common inflammatory pathways.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-025-03439-x.

Supplementary material 1

Author contributions

Xiangxue Li, Zhaojun Ni, Weixiong Shi, Kangqing Zhao, Lei Su, Yanxue Xue and Hongqiang Sun designed the experiment. Xiangxue Li analyzed the study data and wrote the initial draft (including substantive translation). Xiangxue Li, Weixiong Shi, Yanjie Zhang and Lina Liu performed the animal experiments, Kangqing Zhao and Zhaojun Ni conducted the research and investigations, specifically performing the human experiments and data collection. Ying Qin, Jingwen Zhao, Wenjuan Peng assisted in recruitment of participants. Zhaojun Ni, Kangqing Zhao, Zhong Wang, Zhoulong Yu, Xuejiao Gao revised the manuscript and participated in editing, interpretation, and revision. Hongqiang Sun, Yanxue Xue, Lei Su, Jie Shi, Lin Lu and Thomas Kosten performed the conceptualization, supervision and project administration. All authors contributed to and have approved the final manuscript.

Funding

This work was supported in part by the Ministry of Science and Technology of the People's Republic of China (STI2030-Major Projects2021ZD0201900), National Natural Science Foundation of China (81971235), National Clinical Research Center for Mental Disorders (Peking University Sixth Hospital) NCRC2020M09, National Key R&D Program of China(2021YFF0306500), Key Attending Psychiatrist Program of Peking University Sixth Hospital (BDLYYLZL2024-02), National Key R&D Program of China (2021YFF0702900).

Data availability

16S rRNA gene sequence data are available in the Sequence Read Archive (SRA) under BioProject accession PRJNA1127822 and PRJNA1127800.

Declarations

Ethics approval and consent to participate

The human study protocol was approved by the Ethics Committee of Peking University Sixth Hospital. All enrolled subjects signed an informed consent form. All participants provided written informed consent. All animal experiments were strictly carried out in accordance with protocol approved by Institutional Animal Care and Use Committee of Peking University Health Science Center.

Competing interests

The authors declare no competing interests.

Received: 29 January 2025 Accepted: 6 April 2025
Published online: 15 April 2025

References

1. Huang Y, Wang Y, Wang H, Liu Z, Yu X, Yan J, et al. Prevalence of mental disorders in China: a cross-sectional epidemiological study. Lancet Psychiatry. 2019;6(3):211–24.
2. Pratt OE, Rooprai HK, Shaw GK, Thomson AD. The genesis of alcoholic brain tissue injury. Alcohol Alcohol. 1990;25(2–3):217–30.
3. Crews FT, Lawrimore CJ, Walter TJ, Coleman LG Jr. The role of neuroimmune signaling in alcoholism. Neuropharmacology. 2017;122:56–73.
4. Prajapati SK, Bhaseen S, Krishnamurthy S, Sahu AN. Neurochemical evidence of preclinical and clinical reports on target-based therapy in alcohol used disorder. Neurochem Res. 2020;45(2):491–507.
5. Vassallo G, Mirijello A, Ferrulli A, Antonelli M, Landolfi R, Gasbarrini A, et al. Review article: Alcohol and gut microbiota - the possible role of gut microbiota modulation in the treatment of alcoholic liver disease. Aliment Pharmacol Ther. 2015;41(10):917–27.
6. Flores-Bastías O, Karahanian E. Neuroinflammation produced by heavy alcohol intake is due to loops of interactions between Toll-like 4 and TNF receptors, peroxisome proliferator-activated receptors and the central melanocortin system: a novel hypothesis and new therapeutic avenues. Neuropharmacology. 2018;128:401–7.
7. Shi G, Zhong S. Alcohol-associated cancer and deregulation of Pol III genes. Gene. 2017;612:25–8.
8. Topiwala A, Ebmeier KP. Effects of drinking on late-life brain and cognition. Evid-Based Ment Heal. 2018;21(1):12–5.
9. Yalcin EB, McLean T, Tong M, de la Monte SM. Progressive white matter atrophy with altered lipid profiles is partially reversed by short-term abstinence in an experimental model of alcohol-related neurodegeneration. Alcohol. 2017;65:51–62.
10. Tiwari V, Chopra K. Resveratrol abrogates alcohol-induced cognitive deficits by attenuating oxidative-nitrosative stress and inflammatory cascade in the adult rat brain. Neurochem Int. 2013;62(6):861–9.
11. de la Monte SM, Kril JJ. Human alcohol-related neuropathology. Acta Neuropathol. 2014;127(1):71–90.
12. Hanak C, Benoit J, Fabry L, Hein M, Verbanck P, de Witte P, et al. Changes in pro-inflammatory markers in detoxifying chronic alcohol abusers, divided by Lesch typology reflect cognitive dysfunction. Alcohol Alcohol. 2017;52(5):529–34.
13. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov. 2008;7(2):156–67.
14. Weitzberg E, Lundberg JO. Novel aspects of dietary nitrate and human health. Annu Rev Nutr. 2013;33:129–59.
15. Kapil V, Khambata RS, Jones DA, Rathod K, Primus C, Massimo G, et al. The noncanonical pathway for in vivo nitric oxide generation: the nitrate-nitrite-nitric oxide pathway. Pharmacol Rev. 2020;72(3):692–766.
16. Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, et al. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. Nat Med. 1995;1(6):546–51.
17. Presley TD, Morgan AR, Bechtold E, Clodfelter W, Dove RW, Jennings JM, et al. Acute effect of a high nitrate diet on brain perfusion in older adults. Nitric Oxide. 2011;24(1):34–42.
18. Wightman EL, Haskell-Ramsay CF, Thompson KG, Blackwell JR, Winyard PG, Forster J, et al. Dietary nitrate modulates cerebral blood flow parameters and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation. Physiol Behav. 2015;149:149–58.
19. Toda N, Ayajiki K, Okamura T. Cerebral blood flow regulation by nitric oxide: recent advances. Pharmacol Rev. 2009;61(1):62–97.
20. Cauwels A, Buys ES, Thoonen R, Geary L, Delanghe J, Shiva S, et al. Nitrite protects against morbidity and mortality associated with TNF- or LPS-induced shock in a soluble guanylate cyclase-dependent manner. J Exp Med. 2009;206(13):2915–24.
21. Vanhatalo A, L'Heureux JE, Kelly J, Blackwell JR, Wylie LJ, Fulford J, et al. Network analysis of nitrate-sensitive oral microbiome reveals interactions with cognitive function and cardiovascular health across dietary interventions. Redox Biol. 2021;41:101933.
22. Justice JN, Johnson LC, DeVan AE, Cruickshank-Quinn C, Reisdorph N, Bassett CJ, et al. Improved motor and cognitive performance with sodium nitrite supplementation is related to small metabolite signatures: a pilot trial in middle-aged and older adults. Aging. 2015;7(11):1004–21.

23. Shannon OM, Easton C, Shepherd A, Siervo M, Bailey SJ, Clifford T. Dietary nitrate and population health: a narrative review of the translational potential of existing laboratory studies. BMC Sports Sci Med R. 2021. https://doi.org/10.1186/s13102-021-00292-2.
24. Wang SC, Chen YC, Chen SJ, Lee CH, Cheng CM. Alcohol addiction, gut microbiota, and alcoholism treatment: a review. Int J Mol Sci. 2020;21(17):6413.
25. Xu Z, Wang C, Dong XG, Hu T, Wang LL, Zhao WB, et al. Chronic alcohol exposure induced gut microbiota dysbiosis and its correlations with neuropsychic behaviors and brain BDNF/Gabra1 changes in mice. BioFactors. 2019;45(2):187–99.
26. Li X, Zhao K, Chen J, Ni Z, Yu Z, Hu L, et al. Diurnal changes of the oral microbiome in patients with alcohol dependence. Front Cell Infect Microbiol. 2022;12:1068908.
27. Wang Z, Chen WH, Li SX, He ZM, Zhu WL, Ji YB, et al. Gut microbiota modulates the inflammatory response and cognitive impairment induced by sleep deprivation. Mol Psychiatry. 2021;26(11):6277–92.
28. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell. 2013;155(7):1451–63.
29. Wong ML, Inserra A, Lewis MD, Mastronardi CA, Leong L, Choo J, et al. Inflammasome signaling affects anxiety- and depressive-like behavior and gut microbiome composition. Mol Psychiatry. 2016;21(6):797–805.
30. Sharon G, Cruz NJ, Kang DW, Gandal MJ, Wang B, Kim YM, et al. Human gut microbiota from autism spectrum disorder promote behavioral symptoms in mice. Cell. 2019;177(6):1600-18 e17.
31. Zheng P, Zeng B, Zhou C, Liu M, Fang Z, Xu X, et al. Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. Mol Psychiatry. 2016;21(6):786–96.
32. Fan Y, Pedersen O. Gut microbiota in human metabolic health and disease. Nat Rev Microbiol. 2021;19(1):55–71.
33. Dubinkina VB, Tyakht AV, Odintsova VY, Yarygin KS, Kovarsky BA, Pavlenko AV, et al. Links of gut microbiota composition with alcohol dependence syndrome and alcoholic liver disease. Microbiome. 2017. https://doi.org/10.1186/s40168-017-0359-2.
34. Seyedmoalemi MA, Saied-Moallemi Z. Association between periodontitis and Alzheimer's disease: a narrative review. Ibro Neurosci Rep. 2025;18:360–5.
35. Araújo RD, Villoria GEM, Luiz RR, Esteves JC, Leao ATT, Feres EJ. Association between periodontitis and Alzheimer's disease and its impact on the self-perceived oral health status: a case-control study. Clin Oral Invest. 2021;25(2):555–62.
36. Ryder MI. Porphyromonas gingivalis and Alzheimer disease: recent findings and potential therapies. J Periodontol. 2020;91(Suppl 1):S45–9.
37. Holmer J, Eriksdotter M, Schultzberg M, Pussinen PJ, Buhlin K. Association between periodontitis and risk of Alzheimer's disease, mild cognitive impairment and subjective cognitive decline: a case-control study. J Clin Periodontol. 2018;45(11):1287–98.
38. Park SC, Yoon JW, Park W. Cognition and oral health: association between Alzheimer's disease and periodontitis. Psychiat Ann. 2024. https://doi.org/10.3928/00485713-20240722-01.
39. Hernandez BY, Zhu X, Sotto P, Paulino Y. Oral exposure to environmental cyanobacteria toxins: implications for cancer risk. Environ Int. 2021;148:106381.
40. Levert-Levitt E, Shapira G, Sragovich S, Shomron N, Lam JCK, Li VOK, et al. Oral microbiota signatures in post-traumatic stress disorder (PTSD) veterans. Mol Psychiatry. 2022. https://doi.org/10.1038/s41380-022-01704-6.
41. Siddiqui H. High throughput sequencing detect gingivitis and periodontal oral Bacteria in Alzheimer's disease. Neuro Res. 2019;1(1):3.
42. Bala S, Csak T, Saha B, Zatsiorsky J, Kodys K, Catalano D, et al. The pro-inflammatory effects of miR-155 promote liver fibrosis and alcohol-induced steatohepatitis. J Hepatol. 2016;64(6):1378–87.
43. Cordero-Herrera I, Guimaraes DD, Moretti C, Zhuge Z, Han H, McCann Haworth S, et al. Head-to-head comparison of inorganic nitrate and metformin in a mouse model of cardiometabolic disease. Nitric Oxide. 2020;97:48–56.
44. Bescos R, Ashworth A, Cutler C, Brookes ZL, Belfield L, Rodiles A, et al. Effects of Chlorhexidine mouthwash on the oral microbiome. Sci Rep. 2020;10(1):5254.
45. Hendgen-Cotta UB, Luedike P, Totzeck M, Kropp M, Schicho A, Stock P, et al. Dietary nitrate supplementation improves revascularization in chronic ischemia. Circulation. 2012;126(16):1983–92.
46. Petersson J, Carlstrom M, Schreiber O, Phillipson M, Christoffersson G, Jagare A, et al. Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash. Free Radic Biol Med. 2009;46(8):1068–75.
47. Xiao E, Mattos M, Vieira GHA, Chen SS, Correa JD, Wu YY, et al. Diabetes enhances IL-17 expression and alters the oral microbiome to increase its pathogenicity. Cell Host Microbe. 2017;22(1):120.
48. Qiao Y, Gong W, Li B, Xu R, Wang M, Shen L, et al. Oral microbiota changes contribute to autism spectrum disorder in mice. J Dent Res. 2022;101(7):821–31.
49. Sheehan DV, Lecrubier Y, Sheehan KH, Amorim P, Janavs J, Weiller E, et al. The Mini-International Neuropsychiatric Interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. J Clin Psychiatry. 1998;59(Suppl 20):22–33.
50. Sokolowska N, Sokolowski R, Polak-Szabela A, Mazur E, Podhorecka M, Kedziora-Kornatowska K. Comparison of the effectiveness of the Montreal cognitive assessment 7.2 and the Mini-Mental State examination in the detection of mild neurocognitive disorder in people over 60 years of age. Preliminary study. Psychiatr Pol. 2018;52(5):843–57.
51. Yu J, Li J, Huang X. The Beijing version of the montreal cognitive assessment as a brief screening tool for mild cognitive impairment: a community-based study. BMC Psychiatry. 2012. https://doi.org/10.1186/1471-244X-12-156.
52. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75(23):7537–41.
53. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. UniFrac: an effective distance metric for microbial community comparison. Isme j. 2011;5(2):169–72.
54. Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, et al. PICRUSt2 for prediction of metagenome functions. Nat Biotechnol. 2020;38(6):685–8.
55. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335–6.
56. Spear LP. Effects of adolescent alcohol consumption on the brain and behaviour. Nat Rev Neurosci. 2018;19(4):197–214.
57. Lisman J, Buzsáki G, Eichenbaum H, Nadel L, Ranganath C, Redish AD. Viewpoints: how the hippocampus contributes to memory, navigation and cognition. Nat Neurosci. 2017;20(11):1434–47.
58. Kim H, Ahrlund-Richter S, Wang X, Deisseroth K, Carlen M. Prefrontal parvalbumin neurons in control of attention. Cell. 2016;164(1–2):208–18.
59. Voet S, Prinz M, van Loo G. Microglia in central nervous system inflammation and multiple sclerosis pathology. Trends Mol Med. 2019;25(2):112–23.
60. Szabo G, Lippai D. Converging actions of alcohol on liver and brain immune signaling. Int Rev Neurobiol. 2014;118:359–80.
61. Lowe PP, Morel C, Ambade A, Iracheta-Vellve A, Kwiatkowski E, Satishchandran A, et al. Chronic alcohol-induced neuroinflammation involves CCR2/5-dependent peripheral macrophage infiltration and microglia alterations. J Neuroinflammation. 2020;17(1):296.
62. Korfias S, Stranjalis G, Papadimitriou A, Psachoulia C, Daskalakis G, Antsaklis A, et al. Serum S-100B protein as a biochemical marker of brain injury: a review of current concepts. Curr Med Chem. 2006;13(30):3719–31.
63. Riedel F, Goessler UR, Hormann K. Alcohol-related diseases of the mouth and throat. Dig Dis. 2005;23(3–4):195–203.
64. Fan XZ, Peters BA, Jacobs EJ, Gapstur SM, Purdue MP, Freedman ND, et al. Drinking alcohol is associated with variation in the human oral microbiome in a large study of American adults. Microbiome. 2018;6(1):596.
65. Lee TJF. Nitric oxide and the cerebral vascular function. J Biomed Sci. 2000;7(1):16–26.

66. Lees B, Meredith LR, Kirkland AE, Bryant BE, Squeglia LM. Effect of alcohol use on the adolescent brain and behavior. Pharmacol Biochem Behav. 2020;192:172906.
67. Blanco AM, Valles SL, Pascual M, Guerri C. Involvement of TLR4/type I IL-1 receptor signaling in the induction of inflammatory mediators and cell death induced by ethanol in cultured astrocytes. J Immunol. 2005;175(10):6893–9.
68. Lippai D, Bala S, Petrasek J, Csak T, Levin I, Kurt-Jones EA, et al. Alcohol-induced IL-1β in the brain is mediated by NLRP3/ASC inflammasome activation that amplifies neuroinflammation. J Leukoc Biol. 2013;94(1):171–82.
69. O'Keefe J, Dostrovsky J. The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. Brain Res. 1971;34(1):171–5.
70. Nakashiba T, Young JZ, McHugh TJ, Buhl DL, Tonegawa S. Transgenic inhibition of synaptic transmission reveals role of CA3 output in hippocampal learning. Science. 2008;319(5867):1260–4.
71. Guan H, Middleton SJ, Inoue T, McHugh TJ. Lateralization of CA1 assemblies in the absence of CA3 input. Nat Commun. 2021;12(1):6114.
72. Hyde ER, Andrade F, Vaksman Z, Parthasarathy K, Jiang H, Parthasarathy DK, et al. Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: implications for nitric oxide homeostasis. PLoS ONE. 2014;9(3): e88645.
73. Doel JJ, Benjamin N, Hector MP, Rogers M, Allaker RP. Evaluation of bacterial nitrate reduction in the human oral cavity. Eur J Oral Sci. 2005;113(1):14–9.
74. Montenegro MF, Sundqvist ML, Nihlen C, Hezel M, Carlstrom M, Weitzberg E, et al. Profound differences between humans and rodents in the ability to concentrate salivary nitrate: Implications for translational research. Redox Biol. 2016;10:206–10.
75. Jansson EA, Huang L, Malkey R, Govoni M, Nihlen C, Olsson A, et al. A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. Nat Chem Biol. 2008;4(7):411–7.
76. Donald K, Finlay BB. Early-life interactions between the microbiota and immune system: impact on immune system development and atopic disease. Nat Rev Immunol. 2023;23(11):735–48.
77. Kayama H, Okumura R, Takeda K. Interaction between the microbiota, epithelia, and immune cells in the intestine. Annu Rev Immunol. 2020;38:23–48.
78. Harding A, Gonder U, Robinson SJ, Crean S, Singhrao SK. Exploring the association between Alzheimer's disease, oral health, microbial endocrinology and nutrition. Front Aging Neurosci. 2017;9:398.
79. Shoemark DK, Allen SJ. The microbiome and disease: reviewing the links between the oral microbiome, aging, and Alzheimer's disease. J Alzheimer's Dis JAD. 2015;43(3):725–38.
80. Kamer AR, Craig RG, Dasanayake AP, Brys M, Glodzik-Sobanska L, de Leon MJ. Inflammation and Alzheimer's disease: possible role of periodontal diseases. Alzheimers Dement. 2008;4(4):242–50.
81. Wu Z, Ni J, Liu Y, Teeling JL, Takayama F, Collcutt A, et al. Cathepsin B plays a critical role in inducing Alzheimer's disease-like phenotypes following chronic systemic exposure to lipopolysaccharide from Porphyromonas gingivalis in mice. Brain Behav Immun. 2017;65:350–61.
82. Sureda A, Daglia M, Castilla SA, Sanadgol N, Nabavi SF, Khan H, et al. Oral microbiota and Alzheimer's disease: do all roads lead to Rome? Pharmacol Res. 2020;151:104582.
83. Watanabe Y, Arai H, Hirano H, Morishita S, Ohara Y, Edahiro A, et al. Oral function as an indexing parameter for mild cognitive impairment in older adults. Geriatr Gerontol Int. 2018;18(5):790–8.
84. Vanhatalo A, Blackwell JR, L'Heureux JE, Williams DW, Smith A, van der Giezen M, et al. Nitrate-responsive oral microbiome modulates nitric oxide homeostasis and blood pressure in humans. Free Radic Biol Med. 2018;124:21–30.
85. Maldonado RF, Sa-Correia I, Valvano MA. Lipopolysaccharide modification in Gram-negative bacteria during chronic infection. FEMS Microbiol Rev. 2016;40(4):480–93.
86. Chen G, Shaw MH, Kim YG, Nunez G. NOD-like receptors: role in innate immunity and inflammatory disease. Annu Rev Pathol. 2009;4:365–98.
87. Schmidt TS, Hayward MR, Coelho LP, Li SS, Costea PI, Voigt AY, et al. Extensive transmission of microbes along the gastrointestinal tract. Elife. 2019. https://doi.org/10.7554/eLife.42693.
88. Aburto MR, Cryan JF. Gastrointestinal and brain barriers: unlocking gates of communication across the microbiota-gut-brain axis. Nat Rev Gastro Hepat. 2024;21(5):365.

Publisher's Note