

Full-length Article

Probiotic supplementation as a strategy to counteract chronic hypoxia-related damages

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ABSTRACT

Chronic hypoxia plays a critical role in the pathogenesis of numerous diseases by triggering cellular and molecular adaptations that disrupt tissue homeostasis, impair gut eubiosis, promote inflammation, and contribute to progressive functional decline. These effects mimic key features of neurodegenerative diseases and share mechanistic overlap with adverse outcomes of airborne environmental exposures that compromise oxygen homeostasis through mechanisms involving Hypoxia-Inducible Factor 1- α (HIF-1 α) stabilization, proteasome dysfunction, and gut-brain axis disruption.

In this study, C57BL/6 mice were exposed to 12% O₂ for 2 months and received oral probiotics supplementation, which rescued short-term memory deficits in the novel object recognition test, reduced hippocampal HIF-1 α accumulation, restored prolyl hydroxylase domain protein 2 (PHD2) and ubiquitin–proteasome pathway, and attenuated neuroinflammation and apoptosis, while elevating brain-derived neurotrophic factor (BDNF). Probiotics also normalized plasma gut hormones (glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), and leptin), enhanced ileal tight junction proteins (zonulin-1, occludin and claudin) in ileal tissue, countered gut microbiota β -diversity shifts and restored beneficial bacterial metabolites with improved predicted metabolic pathways. These findings provide preclinical evidence that probiotics oral supplementation can restore gut-brain axis homeostasis, and mitigate chronic hypoxia related neuroinflammation, offering a potential therapeutic strategy against neurodegeneration triggered by oxygen-depriving environmental and pathological conditions.

1. Introduction

Hypoxia is a reduction in oxygen availability, forcing tissues and organs to activate adaptive responses (Della Rocca et al., 2022). It may arise from obstructive sleep apnoea, high altitude exposure (Della Rocca et al., 2022), or chronic hypoperfusion, with the hippocampus being especially vulnerable to hypoxia-dependent dysfunction. Chronic hypoxia has attracted growing interest in neurobiology, because it reproduces several features commonly associated with neurodegenerative

disorders, including neuroinflammation, impaired proteostasis, neuronal loss, and cognitive decline (Önder, 2025; Wolters et al., 2017) (Zhang and Le, 2010) (Lestón Pinilla et al., 2021).

At the molecular level, hypoxia-inducible factors (HIF) orchestrate cellular and systemic adaptation to hypoxic stimuli (Della Rocca et al., 2022). A key player is HIF-1 α , which is constitutively expressed and, is promptly targeted for proteasomal degradation by the action of prolyl hydroxylase domain protein 2 (PHD2) (Della Rocca et al., 2022) at physiological oxygen concentrations. However, when O₂ levels fall

Abbreviations: BDNF, brain-derived neurotrophic factor; CNS, the central nervous system; EPO, erythropoietin; GIP, glucose-dependent insulinotropic polypeptide; GM, gut microbiota; HIF-1 α , Hypoxia-Inducible Factor 1- α ; Iba-1, ionized calcium-binding adapter molecule 1; IL-1, Interleukin-1 β ; NOR, Novel Object Recognition; PD, Parkinson's disease; AD, Alzheimer's disease; PHD2, prolyl hydroxylase domain protein 2; RBCs, red blood cells; SCFAs, Short-chain fatty acids; ZO-1, Zonulin-1.

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below a critical threshold, PHD2 activity is impaired, allowing HIF-1 α accumulation, nuclear translocation, and transcriptional activation of hypoxia-responsive genes (Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1 α is mediated by an O₂-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci. Proceedings of the National Academy of Sciences*; 1998). Under hypoxia, stabilized HIF-1 α transcriptionally activates critical adaptive pathways, such as erythropoietin (EPO)-mediated erythropoiesis (Burtscher et al., 2022) and GLUT-1-dependent glucose transport (Zhang et al., 1999). Notably, EPO levels peak early but plateau within 72h of sustained hypoxia exposure. (Vizcardo-Galindo et al., 2020; Saugy et al., 2022). At first glance, adaptations triggered by HIF-1 α accumulations are beneficial, as they allow an efficient cellular and physiological reaction to the negative stimulus (Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1 α is mediated by an O₂-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci. Proceedings of the National Academy of Sciences*; 1998), however, upon chronic exposure to oxygen deprivation the subsequent cellular response triggers a series of detrimental effects on tissues and organs (Pham et al., 2021; Zhao et al., 2022; Ma et al., 2024; NLRP3, 2023; Lu et al., 2025).

Hypoxia and neurodegeneration share key cerebral features, such as neuroinflammation, decrease in neurotrophic factor expression, neuronal loss, and impairments in proteasome activity (Wolters et al., 2017). Hypoxia has also been found to influence gut microbiota (GM) (Han et al., 2021). Specifically, high-altitude and consequent hypoxia exposure have been reported to trigger a reduction in α - and β -diversity of the GM (Acute exposure to simulated high-altitude hypoxia alters gut microbiota in mice | *Archives of Microbiology* [Internet]. [cited, 2025], as well as to induce a compositional shift toward bacterial species generally associated with negative effects, leading to a condition called gut dysbiosis (Kumari et al., 2025). One of the most common outcomes of dysbiosis is the disruption of the gut barrier (Marcari et al., 2025). Perturbation of the physiological GM composition may compromise gut barrier integrity (Marcari et al., 2025), leading to increased leakage of bacterial toxins and food particles into the systemic circulation, which promotes systemic inflammation, blood–brain barrier (BBB) disruption, and ultimately neurodegeneration with cerebral hypometabolism, and general energetic dyshomeostasis (Bonfili et al., 2017). Such changes modulate the systemic release of appetite-regulating hormones (Obradovic et al., 2021), including enteroendocrine-derived glucagon like peptide 1 (GLP-1) and glucose insulinotropic polypeptide (GIP), and adipocyte-secreted leptin, which collectively play a critical role in energy expenditure and food intake (Obradovic et al., 2021; Baggio and Drucker, 2007; Smith et al., 2019). These alterations modify the release of gut-derived mediators, including short-chain fatty acids (SCFAs) and enteroendocrine hormones, both of which can influence brain physiology and neuroimmune homeostasis (Kim et al., 2019).

This makes the gut-brain axis a plausible mechanistic route linking chronic hypoxia to hippocampal dysfunction. Interestingly, GM offers a potential therapeutic target, and probiotics represent an effective and non-invasive way to modulate GM composition (Kim et al., 2019; Sarita et al., 2025).

Previous work with the same multistrain formulation used here showed beneficial effects in a mouse model of Alzheimer's disease (3xTg-AD) by restoring gut–brain axis homeostasis (Bonfili et al., 2017). Specifically, it demonstrated effectiveness in reducing amyloid load, cognitive decline, neuroinflammation, and neuronal loss, while also reducing brain hypometabolism (Bonfili et al., 2017; Bonfili et al., 2022; Bonfili et al., 2018; A red lentils-based synbiotic cookie exerts neuroprotective effects in a mouse model of Alzheimer's disease, 2025).

The same probiotic formulation triggered the expression of HIF-1 α in 3xTg-AD mouse brain, possibly indicating enhanced angiogenesis, which could be linked to the rescue of brain hypometabolism (Bonfili et al., 2022). Notably, this probiotic formulation restored blood oxygen saturation in hypoxemic preterm infants (Baldassarre et al., 2023)

and hospitalized COVID-19 patients (Ceccarelli et al., 2021), corroborating an oxygen-sparing mechanism.

However, it remains unclear whether a similar approach can attenuate the complex intestinal and cerebral alterations induced by sustained hypoxia. In particular, the literature lacks an integrated analysis of chronic hypoxia combining behaviour, hippocampal hypoxia-responsive pathways, gut barrier markers, circulating gut-derived mediators, and microbiota remodeling within the same preclinical paradigm.

The present study was designed to address this gap. Specifically, four groups of C57BL/6 mice were established: two normoxic groups (with or without probiotic supplementation) and two hypoxic groups (12% O₂), with or without probiotic administration. After 2 months of treatment, cognitive function was assessed by Novel Object Recognition and Open Field tests, while hippocampal hypoxic signaling (HIF-1 α , PHD2, EPO, GLUT1), neuroinflammation, apoptosis, and proteasome activity were analysed. In parallel, gut microbiota remodelling was profiled by 16S rRNA sequencing, and systemic SCFAs, gut hormones, and gut and brain barrier integrity were evaluated. The aim was to determine whether probiotic treatment is associated with a coordinated protection of gut-brain axis homeostasis during chronic hypoxic stress.

2. Material and methods

2.1. Reagents and chemicals

The multistrain probiotic formulation included eight lyophilized bacterial strains of lactic acid bacteria and bifidobacteria: *Streptococcus thermophilus* CNCM I-5570, *Lactobacillus brevis* CNCM I-5566 *Bifidobacterium animalis subsp. lactis* CNCM I-5571, *Bifidobacterium animalis subsp. lactis* CNCM I-5572, *Lactobacillus plantarum* CNCM I-5569, *Lactobacillus paracasei* CNCM I-5568, *Lactobacillus acidophilus* CNCM I-5567, *Lactobacillus helveticus* CNCM I-5573 (OXXYSLABTM, manufactured by EOS2021 Srl, Ardea, Rome, Italy, formerly SLAB51®).

Polyvinylidene difluoride (PVDF) membranes were obtained from Merck KGaA (Darmstadt, Germany). Reagents for western blotting analyses were obtained from Biorad (Milano, Italy). Antibodies for detecting hypoxia inducible factor (HIF-1 α ; H6536), prolyl hydroxylase domain-containing protein 2 (PHD2; ab2268890), inducible nitric oxide synthase (iNOS; ab3523), ionized calcium-binding adapter molecule 1 (Iba-1; ab178846) and interleukin-1 β (IL-1; ab9722), were purchased from AbCam (Milan, Italy). Bcl-2 (sc-7382), BAX (sc-70408) and glial fibrillary acidic protein (GFAP; sc-33673) were from Santa Cruz Biotechnology.

Proteases inhibitors tosyl phenylalanyl chloromethyl ketone (TPCK) and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF or Pefabloc) were obtained from Sigma-Aldrich S.r.L. (Milano, Italy). All solvents and reagents used in this experiment were of the highest purity available.

2.2. Animal model

C57BL/6 mice were housed in plastic cages (Tecniplast SpA, Buguggiate, VA, Italy), 4 animals per cage, in a temperature-controlled room (21 $\pm$ 5 °C), 60% humidity, 12 h light/dark inverted cycle (light was switched on at 8:00 PM) and were fed laboratory mice diet (Mucedola Srl, Settimo Milanese, MI, Italy) with water ad libitum. Eight-week-old mice (weight 15–25 g) were used for the experiments. All experimental procedures were approved by the Italian Ministry of Health (authorization n° 394/2024-PR), following the national legislation (Legislative Decree 26/2014) and the European Communities Council Directives (2010/63/EU). Appropriate measures minimized pain and discomfort in experimental animals.

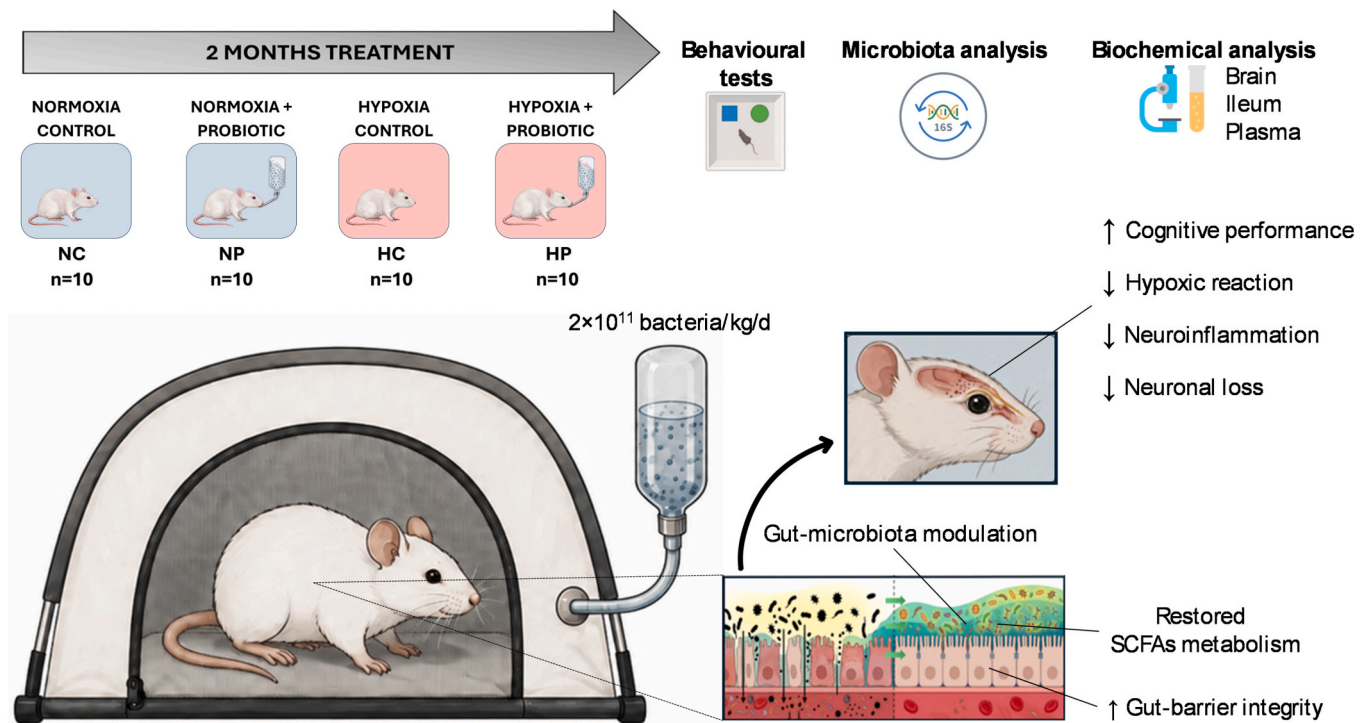


Fig. 1. Experimental design. Eight-week-old C57BL/6 gender-balanced mice were assigned to four groups: normoxia control (NC, $n = 10$) housed at $\sim 21\%$ O_2 ; normoxia probiotic (NP, $n = 10$) receiving probiotics (2×10^{11} bacteria/kg/day) in drinking water under normoxia; hypoxia control (HC, $n = 10$) housed under 12% O_2 generated by a Hypoxico “Sierra 100” altitude system; and hypoxia probiotic (HP, $n = 11$) exposed to 12% O_2 and receiving probiotics. After 2 months of treatment, behavioural tests were performed and plasma, faeces, brain, and ileum samples were collected for microbiota analysis and biochemical assays. The potential mechanisms explored in the study are presented.

2.3. Experimental design

Eight-week-old C57BL/6 gender balanced mice were randomly allocated into four groups: 1) control normoxia group (NC, $n = 10$), in which animals were housed at standard atmospheric oxygen concentrations ($\sim 21\%$ O_2); 2) normoxia probiotic group (NP, $n = 10$) with animals kept at normoxic conditions receiving probiotics (2×10^{11} bacteria/kg/d), freshly dissolved in drinking water and replaced daily following preliminary verification of strain viability in aqueous suspension; 3) hypoxia control group (HC, $n = 10$), with animals kept under an hypoxic tent equipped with a Hypoxico “Sierra 100” altitude generator (Hypoxico Inc, Gardiner, NY, USA) that precisely modulate oxygen concentration (Wright et al., 2021), producing an environment with 12% oxygen; 4) hypoxia probiotic group (HP, $n = 10$) with animals kept under the mentioned hypoxic conditions and receiving the probiotic formulation, as the NP group. Animals were treated for a period of 2 months (Fig. 1). The 2×10^{11} bacteria/kg/day dose was selected based on prior studies demonstrating optimal efficacy and viability of this specific multistrain formulation in mouse models of neurodegeneration (Bonfili et al., 2017; Bonfili et al., 2025).

This multistrain strategy is particularly relevant because cooperative interactions among consortium members may create a favorable microenvironment that supports the growth and functional activity of other strains, an emergent property that cannot be captured by testing single strains in isolation. Blinding procedures were used for behavioral scoring and image analysis.

2.4. Behavioural assessments

Three days before the sacrifice, the novel object recognition (NOR) test was performed in a plexiglass arena (45Lx45Wx60H cm) with a white floor and opaque walls. The experiment consisted in three phases: habituation, training and test. All phases were performed during the

dark phase under dim light conditions. Before each phase, mice were habituated to the test room for 5 min in individual cages. All phases and all arenas were recorded with a Panasonic USB camera from top view. Experimental data were collected and analyzed with Ethovision XT 7 software (Noldus, Netherlands). During habituation, mice were placed in the centre of the arena and left free to explore it for 10 min. The training phase was performed 24 h after habituation. Mice were placed in the same arena with two identical objects positioned at the opposite angles of the arena and left free to explore for 10 min. The objects were the same for all animals. The time spent exploring each object was measured off-line by two blinded researchers who agreed upon scoring rules beforehand. “Exploration” was considered as the animal’s nose being within a 2-cm radius of the object with the head directed toward it. Time spent sitting on the object or leaning on the object was not counted as exploration unless the head was directed towards the object. Time was measured using the Behavioral Observation Research Interactive Software (BORIS), version 8.22.14 REF: 10.1111/2041-210X.12584. The test phase was performed 3 h after the training phase. Mice were placed in the same arena with two objects, one identical to the training phase and the other novel. Objects were placed at the opposite angles of the arena, and their position was the same for all animals. Mice were left free to explore for 5 min and the time spent exploring each object was measured offline. Exploration definition was the same of the training phase. Time was collected using the BORIS software. Discrimination index was calculated as the ratio of the time spent exploring the novel object and the total time of exploration of the two objects, expressed in percentage.

2.5. Tissue and plasma collection and processing

Upon the 2-month treatment and following the behavioral assessment, mice were euthanized at 24 weeks of age by cervical dislocation. Blood samples were immediately extracted from the cardiac chambers

using a heparinized syringe fitted with a heparinized 26G needle, collected in EDTA-tubes, and centrifuged at 3,500 rpm for 10 min at 4 °C. The plasma supernatant was immediately supplemented with protease inhibitors (1 mM Pefabloc) and stored at -80 °C. Brain tissues were properly collected for subsequent biochemical analyses. In detail, micro-dissected hippocampal and cortical regions of each mouse were flash-frozen in dry ice and stored at -80 °C until use. Tissues were homogenized in 50 mM Tris buffer, 150 mM KCl, 2 mM EDTA, and pH 7.5 (1:5 weight/volume of buffer), centrifuged at 13,000 × g for 20 min at 4 °C, the supernatants were collected and proteinase inhibitors (1 mM TPCK and Pefabloc) were added and stored at -80 °C. Protein concentration was determined using the Bradford protein assay (Schneider et al., 2012).

2.6. Hormones determination

Plasmatic concentration of erythropoietin (EPO) was measured using the Mouse EPO ELISA kit (Antibodies.com Cat# A79298). Ghrelin, leptin, GIP, GLP-1 were determined by ELISA kits from Merck-Millipore (Milan, Italy) respectively, following the manufacturer's instructions. Data are expressed as mean values in pg/mL ± SD.

2.7. Western blotting

HIF-1α, PHD2, ubiquitin conjugates, Iba-1, iNOS, IL-1β, GFAP, Bcl-2, BAX, BDNF, claudin, zonulin-1, and occludin were analyzed through western blot. In detail, 30 µg total protein of brain homogenates were loaded on 10-12% sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto PVDF membranes. After incubation with the specific antibodies, the immunoblot detection was carried out with an ECL western blotting ChemiDocTM System (Biorad, Milan, Italy). Each gel contained molecular weight markers ranging from 6.5 to 205 kDa. β-actin was utilized to guarantee equal protein loading and to normalize western blot data. ImageJ software was used for the densitometric analysis of the western blot results.

2.8. Microbiota analysis

Three days before sacrifice, faecal samples were collected from all groups of mice and immediately stored at -80 °C until processing. For the characterization of the gut microbiota, total DNA was extracted from each collected stool sample using the Stool Mini kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. To increase the DNA yield from Gram-positive bacteria, the standard QIAGEN protocol was modified by incorporating an additional preliminary lysis step: samples were treated with lysozyme (20 mg/mL) for 12 h to enhance bacterial cell wall lysis. The concentration and purity of the extracted DNA were determined using a Bio-Photometer® spectrophotometer (Eppendorf, Milan, Italy) by measuring the absorbance at 260nm and calculating the A₂₆₀/A₂₈₀ ratio. DNA integrity was confirmed via electrophoresis on a 1% agarose gel stained with SYBR™ Safe DNA Gel Stain.

The variable region V3–V4 of the 16S rRNA gene (approximately 460 bp in length) was amplified using the primer pairs: 16S_F5' - (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG) - 3' and 16S_5' - (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C) - 3' as described in the MiSeq rRNA Amplicon Sequencing protocol (Illumina, San Diego, California, USA). The Polymerase Chain Reaction (PCR) was performed using the KAPA Hifi HotStart Ready Mix kit (KAPA Biosystems Inc., Wilmington, Massachusetts, USA), according to the manufacturer's protocol. The resulting DNA amplicons were purified using AMPure XP beads (Beckman Coulter Inc., Beverly, Massachusetts, USA). A second amplification step was then conducted to attach unique dual-indexed bar-coded Illumina Nextera forward and reverse adaptor-primers. The final library

was again cleaned up using 50 µL of AMPure XP beads, quantified using the QuantiT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and normalized to equimolar concentrations (4 nmol/L). The normalized samples were pooled before sequencing on the Illumina MiSeq™ platform to generate 300-base paired-end reads, following the manufacturer's specifications.

For the sequence processing and quality filtering, after demultiplexing, paired reads were merged using USEARCH v11 were removed using Cutadapt 2.1 (Martin, 2011). Sequences were then filtered to retain only those with a total expected error ≤ 0.8 and a length in the range of 400–460 bp.

2.9. Determination of Amplicon Sequence Variants (ASVs) and Taxonomic Assignment

Quality-filtered sequences were imported into the software package QIIME 2 (Quantitative Insights Into Microbial Ecology 2), version 2025.10 (Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2 | Nature Biotechnology [Internet]. [cited, 2026] and processed with the DADA2 algorithm (DADA2: High-resolution sample inference from Illumina amplicon data | Nature Methods [Internet]. [cited, 2026] for chimera checking and the determination of Amplicon Sequence Variants (ASVs). To minimize artifacts, ASVs found in only one sample and/or representing fewer than 10 sequences across the entire population were filtered out. Taxonomic assignment of the ASVs was performed using a Naive Bayes classifier trained on a custom 97-clustered version of the Greengenes rDNA v13.8 reference database, specifically trimmed to include only the V3–V4 regions. ASVs not identified at the species level and/or identified with a confidence score < 0.75 using the reference database were assigned to the deepest possible taxonomic level based on BLAST results obtained by querying the sequences against available published data. Only BLAST results consistent with the taxonomy assigned by the Naive Bayes classifier (Marazzato et al., 2020) (if a confidence score > 0.75 was determined) were accepted, as previously described. The top 20 ASVs and their taxonomic assignments are reported in Supplementary Table 1.

Beta-diversity (diversity between samples) was assessed using the Bray-Curtis dissimilarity measure. Functional metagenomes were predicted from the 16S rRNA reads using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved State 2 (PICRUST2) (Douglas et al., 2020), providing predictive functional profiles.

Statistical analyses were performed using R (version 4.4.1). The permutational multiple analysis of variance (PERMANOVA) test was calculated in QIIME2 with 1,000 permutations on the beta-diversity distance matrices to assess the presence of statistically significant partitions among the groups and between different time points for each group. For each time point considered, the Aldex2 algorithm was used to determine statistically significant differences in microbial composition among the groups. To account for multiple comparisons and decrease the False Discovery Rate (FDR), the computed p-values were corrected using the Benjamini-Hochberg procedure. The significance level for all statistical tests was set at a p-value ≤ 0.05.

Raw sequencing data are available in the NCBI Sequence Read Archive under BioProject accession PRJNA1467165.

2.10. Histology and Immunohistochemistry

After tissue excision, brain and intestinal (ileum) tissue samples were immediately fixed in 10% neutral-buffered formalin. After 24 h, tissues were dehydrated through a graded series of alcohols, cleared with ethanol/xylene solution and embedded in paraffin wax. Serial sections (3 µm-thick) were cut from paraffin-embedded tissue blocks and mounted on polarized slides.

Immunohistochemistry was performed using avidin-biotin-peroxidase complex method (Vectastain Elite ABC Kit, Vector Laboratories Inc., Newark, CA, USA) with 3,3'-diaminobenzidine (Vector

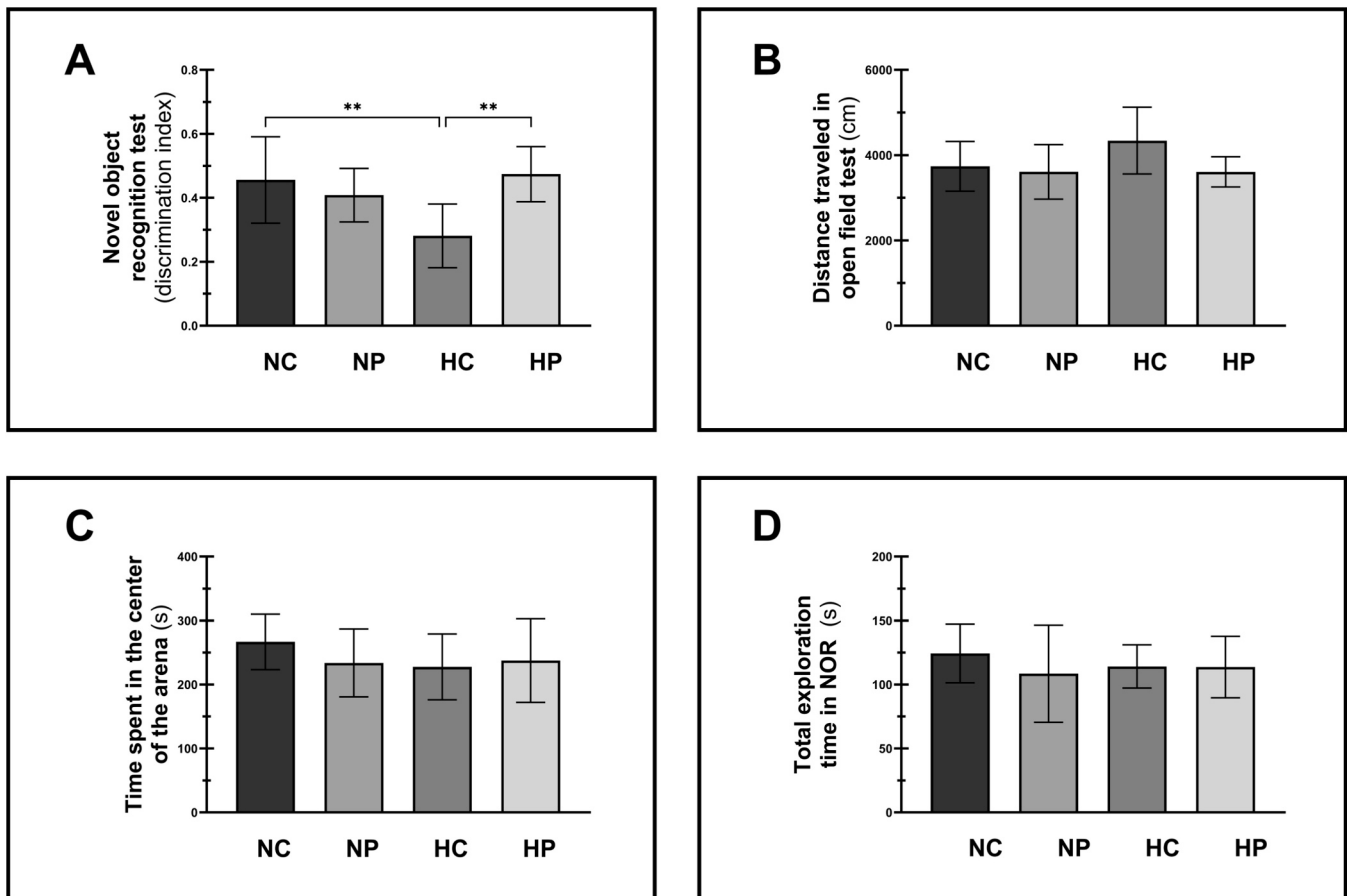


Fig. 2. Probiotics counteracted hypoxia-related memory deficits in mice. Panel A: Hypoxic control discrimination score (time spent in the new object/time spent on both objects) is significantly different from normoxia control and hypoxia probiotic. Data points marked with a single asterisk (*) are statistically significant ($p < 0.05$), while double asterisks (**) indicate $p < 0.001$, as determined by one-way ANOVA followed by Tukey's post hoc test. Panel B, C and D: Total distance travelled in open field test (OF), time spent in the centre of the arena and total exploration time during NOR are provided to report no significant alterations of mice behaviour.

Laboratories Inc., Newark, CA, USA) as chromogen.

The following primary antibodies were used to evaluate tight junction protein expression in intestinal samples: anti-ZO-1 (diluted 1:1000, ab221546, Abcam, Cambridge, UK), anti-occludin (diluted 1: 200, ab216327, Abcam, Cambridge, UK) and anti-claudin 1 (diluted 1:100, MH25- 71-7800, Thermo Fisher Scientific Inc., USA).

For brain sections, an antibody against ionized calcium-binding adaptor molecule 1 (Iba1) was used as a pan-macrophage/microglial marker (diluted 1:300, 019-19741, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), while anti-glial fibrillary acidic protein (GFAP) was employed as a marker for astrocytes (diluted 1:300, Z0334, Agilent Technologies Italia SpA, Cernusco sul Naviglio, MI, Italy). In addition, the expression of hypoxia-inducible factor-1 α (HIF-1 α) was investigated as described above, while for evaluation of the apoptotic rate a TUNEL colorimetric staining (DeadEnd, Promega®) was used according to the manufacturer's instructions. Finally, to evaluate the total number of apoptotic cells, tissue sections were stained using the DeadEnd™ Colorimetric Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) System (Promega Italia Srl, Milano, Italy).

2.11. Immunohistochemical scoring method

For all markers analysed in brain samples, five microscopic fields per section were evaluated using a light microscope (Carl Zeiss, Jena, Germany) equipped with a $\times 40$ objective, a $\times 10$ eyepiece, and a square eyepiece graticule (10×10 squares; total area $62,500 \mu\text{m}^2$). Positive cells were counted in each field, and arithmetic mean values were

calculated.

Tight junction protein expression was assessed using the semi-quantitative scoring method proposed by de Gooyer et al (de Gooyer et al., 2020) which combines both the extent of biomarker expression and staining intensity. Briefly, positive cells were counted in five microscopic fields at $\times 40$ magnification. The number of positive cells was normalized to the total number of cells per field to obtain a mean percentage of positive cells per microscopic field. These percentages were categorized using a 0–4 scale: 0 = no positive cells, 1 = $< 10\%$, 2 = $10 - 50\%$, 3 = $51 - 80\%$, and 4 = $> 80\%$.

The immunoreactive score (IRS) was used for semi-quantitative assessment of staining, by multiplying staining intensity by the proportion of immunopositive cells. For the same microscopic fields, staining intensity was independently evaluated and classified using a 0–3 scale (0 = absent, 1 = mild, 2 = moderate, 3 = intense). The intensity score and percentage score were multiplied to obtain the final IRS, ranging from 0 to 12. Mean IRS values were used for comparisons among experimental groups (de Gooyer et al., 2020).

2.12. Proteasome activity

The 26S proteasome ChT-L activity was tested using Suc-Leu-Leu-Val-Tyr-AMC as substrate and 50 mM Tris/HCl pH 8.0 buffer containing 10 mM MgCl_2 , 1 mM dithiothreitol and 2 mM ATP. The incubation mixture contained 10 μg of tissue lysates, the synthetic substrate and 50 mM Tris/HCl pH 8.0, up to a final volume of 100 μL . Incubation was performed at 37°C , and after 60 min the fluorescence of the hydrolysed

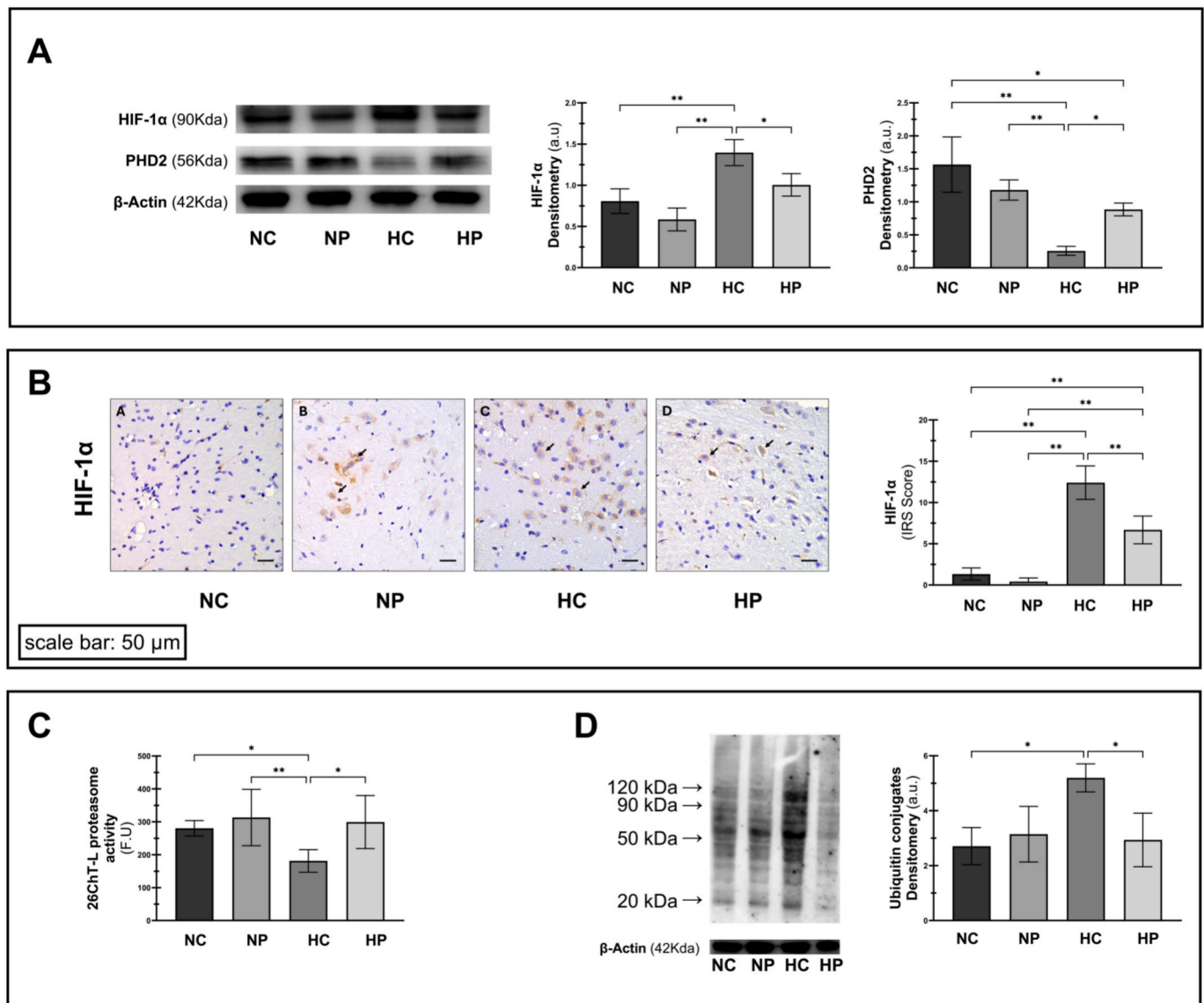


Fig. 3. Probiotic Treatment Modulates Hippocampal HIF-1 α Stabilization and Proteostasis Under Hypoxia. Panel A: Western blot analysis of hippocampal tissue for HIF-1 α and PHD2, the densitometry from three separate blots and a representative immunoblot are reported. Equal protein loading was verified by using an anti- β -actin antibody. Panel B: Representative immunostaining for HIF-1 α in hippocampal sections (scale bars: 50 μ m), quantified as immunoreactivity score (IRS) and expressed as mean $\pm$ SD). Panel C: 26ChT-L activity assay in hippocampal homogenates, expressed as fluorescence units (F.U.). Panel D: Western-blot analysis of ubiquitin (Ub) conjugates of hippocampal homogenates. The densitometry from three separate blots and a representative immunoblot are reported. Equal protein loading was verified by using an anti- β -actin antibody. Data points marked with a single asterisk (*) are statistically significant ($p < 0.05$), while double asterisks (**) indicate $p < 0.001$, as determined by one-way ANOVA followed by Tukey's post hoc test.

AMC was detected (AMC, $\lambda_{exc} = 365$ nm, $\lambda_{em} = 449$ nm) on a SpectraMax Gemini XPS microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.13. Circulating SCFAs determination

Plasmatic concentration of was measured using the Mouse Short-chain fatty acids (SCFAs) ELISA Kit (MyBioSource, Vancouver, Canada) following the manufacturer's instructions. Data are expressed as mean values in pg/mL $\pm$ SD.

2.14. Microbiota-SCFA correlation and heatmap generation

Kendall's rank correlation coefficients (τ) were calculated between the relative abundance of individual bacterial species and plasma SCFA concentrations (ng/mL) independently for each group. Analyses were

orchestrated using a custom Python script that interfaced with R software (v4.6.0) via the rpy2 library, and heatmap visualization was generated using the R pheatmap package. Only species exhibiting a strong correlation ($|\tau| \geq 0.5$) in at least one of the four groups were retained in the heatmap. Asterisks denote statistically significant correlations ($p < 0.05$). Color gradients represent the magnitude and direction of the τ correlation coefficients.

2.15. Statistical analysis

Sample size was determined a priori using G*Power (version 3.1.9.7) based on effect sizes from pilot data and the literature. Achieved statistical power was calculated by post-hoc analysis using G*Power ($>75\%$ post-hoc power for primary outcomes was observed, Supplementary table 2). Gender-balanced design used (~ 5 /sex/group) precluded sex-stratified analyses and composite group analyses were

performed.

Biochemical data are presented as mean values $\pm$ SD ($n = 10$ mice per group). Data were analyzed using one-way ANOVA with group as a categorical variable. Where ANOVA revealed significant differences ($p < 0.05$), a post-hoc Tukey HSD test was performed. The Benjamini–Hochberg false discovery rate (FDR) correction was used to account for multiple hypothesis testing when necessary. In all cases, a p -value ≤ 0.05 was considered statistically significant. Statistical analyses were performed using Sigma-stat 3.1 software (SPSS, Chicago, IL, USA).

3. Results

3.1. Probiotic intervention alleviates hypoxia-induced cognitive decline

Chronic hypoxia promotes neurodegeneration, leading to impaired cognitive function (Ma et al., 2024). In this study, short-term memory was explored using the novel object recognition (NOR) test (Antunes and Biala, 2012). During the habituation phase, the open field test (OF) was conducted to track mouse behaviour (Prut and Belzung, 2003). OF evaluations included time spent in the centre of the arena, commonly used as an index of anxiety-like behaviour, and total distance travelled, which reflects locomotor function and general activity. As shown in Fig. 2, panels B and C, neither the time spent in the centre nor the total distance travelled was affected by hypoxia or probiotic administration, indicating no alterations in anxiety nor locomotion that could influence NOR performance. In contrast, in the novel object recognition test (Fig. 2, panel A), a decrease in short-term memory function was observed in the hypoxic control group, consistent with the neurodegenerative effects of oxygen deprivation. Notably, although probiotic supplementation did not produce any baseline effect in normoxic groups, mice receiving probiotic treatment under hypoxia exhibited a significantly higher discrimination score than the hypoxic control group, suggesting a protective effect of the formulation against hypoxia-induced memory loss. Notably, total exploration time during the NOR training phase remained comparable across groups (Fig. 2, panel D), indicating that the observed deficits in recognition memory were not confounded by general behavioural abnormalities, but were specifically attributable to short-term memory impairment (Fig. 2).

3.2. Modulation of hypoxia-responsive pathways

Following hypoxia induction, the body initiates adaptive responses to the scarce oxygen availability (Cellular adaptation to hypoxia through hypoxia inducible factors and beyond | Nature Reviews Molecular Cell Biology [Internet]. [cited, 2025]). As shown in Fig. 3, panel A, exposure to hypoxia promotes HIF-1 α stabilization in the mouse brain, causing a marked increase in its cerebral levels in the hypoxic groups, which was significantly attenuated by probiotic supplementation. Immunohistochemical staining of hippocampal sections confirmed this pattern (Fig. 3, panel B).

Under normoxic conditions, HIF-1 α degradation relies on the action of PHD2, which hydroxylates specific proline residues on HIF-1 α creating a binding site for HIF-1 α ubiquitination and proteasome mediated degradation (Salceda and Caro, 1997). Accordingly, decreased PHD2 cerebral levels were observed in mice subjected to oxygen restriction. Probiotic supplementation partially counteracted PHD2 reduction during hypoxia (Fig. 3, panels A and B), suggesting a potential regulatory role of probiotics in maintaining oxygen-sensing homeostasis.

During hypoxia proteasome impairment could contribute to stabilize HIF-1 α by inhibiting its oxygen-dependent degradation via the ubiquitin-proteasome pathway. This stabilization correlates with reduced 26S proteasomal activity observed in hypoxic control group (Fig. 3, panel C). Impairments in proteasome activity are frequently reported in neurodegenerative diseases (Thibaut et al., 2018; McNaught KStP, Jenner P., 2001), which are characterized by an

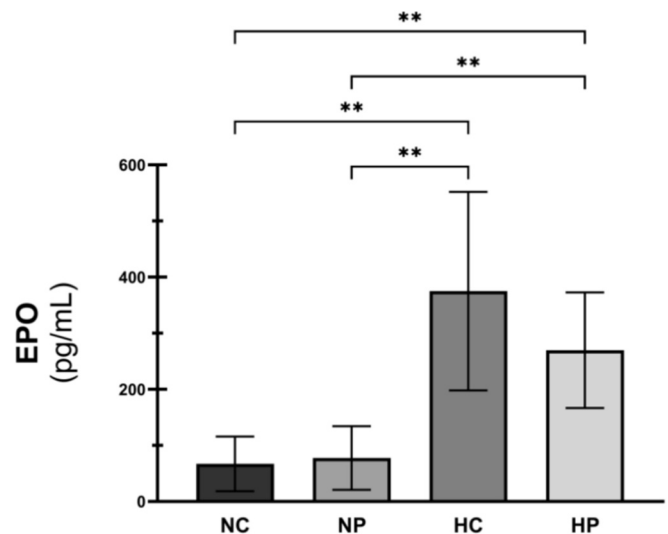


Fig. 4. Probiotics partially prevented EPO overexpression under hypoxic conditions. Erythropoietin plasma concentration (pg/mL) in mice exposed to hypoxia with or without probiotic treatment. Data represent mean $\pm$ SD ($n = 10$). Data points marked with a single asterisk (*) are statistically significant ($p < 0.05$), while double asterisks (**) indicate $p < 0.001$, as determined by one-way ANOVA followed by Tukey's post hoc test.

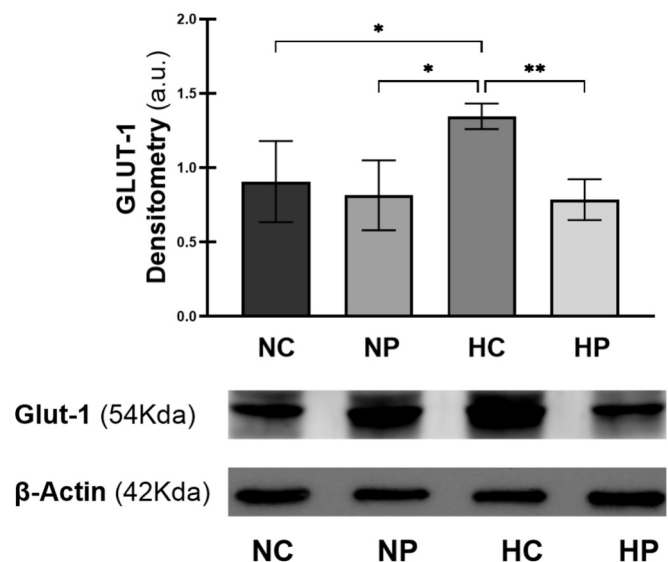


Fig. 5. Cerebral GLUT-1 protein expression. The densitometry from three separate western blots and a representative immunoblot are reported. Equal protein loading was verified by using an anti- β -actin antibody. Data points marked with a single asterisk (*) are statistically significant ($p < 0.05$), while double asterisks (**) indicate $p < 0.001$, as determined by one-way ANOVA followed by Tukey's post hoc test.

accumulation of proteins that is often paired with a reduced proteasomal functionality (Ross and Poirier, 2004). Hypoxia-induced 26S ChT-L activity reduction was accompanied by accumulation of ubiquitin conjugates (Fig. 3, panel C). Notably, these alterations were markedly reduced in the hypoxia group that received the probiotic supplementation. Interestingly, also for these tests, probiotics did not induce any differences in the normoxic groups, reinforcing the hypothesis that the beneficial effects are implemented only in a context of dysbiosis or hypoxic insult.

Among the factors involved in physiological adaptation to hypoxia, upregulation of erythropoietin (EPO) plays a central role.

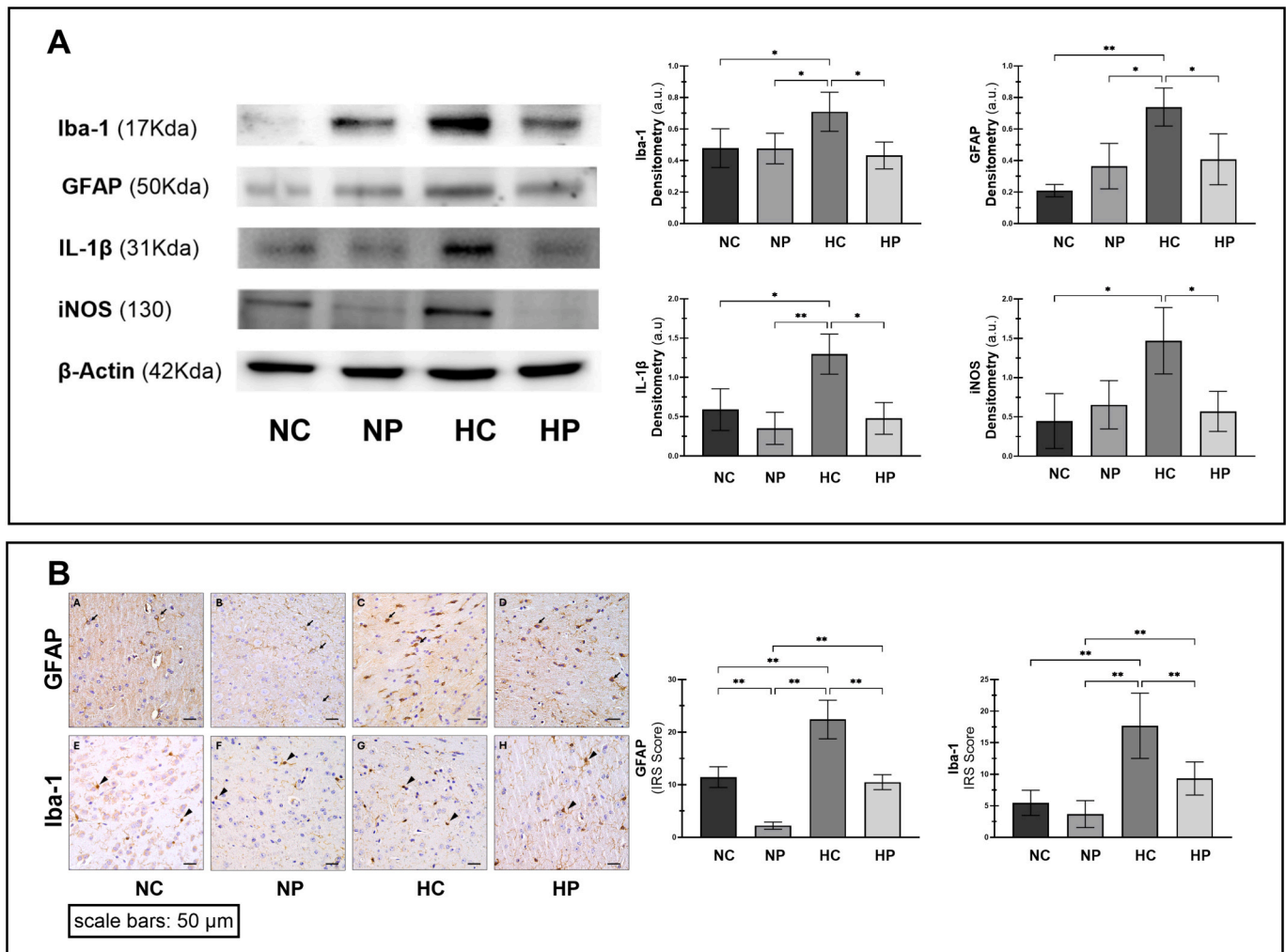


Fig. 6. Probiotics counteract hypoxia-induced neuroinflammation on mice hippocampus. Panel A: Western-blot analysis of hippocampal tissue for inflammatory markers Iba-1, GFAP, IL-1 β and iNOS. The densitometry from three separate blots and a representative immunoblot are reported. Equal protein loading was verified by using an anti- β -actin antibody. Panel B: Representative immunostaining for GFAP and Iba-1 in hippocampal sections (scale bars: 50 μ m), quantified as immunoreactivity score (IRS) and expressed as mean $\pm$ SD. Data points marked with a single asterisk (*) are statistically significant ($p < 0.05$), while double asterisks (**) indicate $p < 0.001$, as determined by one-way ANOVA followed by Tukey's post hoc test.

EPO is a key hypoxia-inducible factor-1 α (HIF-1 α) target gene that stimulates erythropoiesis, and increased plasma EPO levels are commonly reported following short-term hypoxic exposure. Interestingly, EPO levels typically peak within the first days of hypoxia and, while remaining elevated compared to normoxic conditions, progressively decline over time as the organism adapts to chronic hypoxia.

In this context, oral probiotic supplementation (HP) resulted in decreased plasma EPO levels compared to the hypoxic control group (Fig. 4), suggesting an improved physiological adaptation to hypoxic stress, likely by modulating gut microbiota to reduce intestinal permeability and systemic inflammation, thereby mitigating hypoxia-inducible signalling via the gut-brain axis.

As a downstream consequence of HIF-1 α stabilization, glucose transporter GLUT-1 protein levels were significantly elevated in hypoxic brains (Fig. 5), ensuring adaptive glucose uptake under low-oxygen conditions. Probiotic supplementation restored GLUT-1 levels.

3.3. Treatment attenuated neuroinflammatory responses under hypoxic conditions

Because of its high vulnerability to oxygen deprivation, which demands robust GLUT1-dependent glucose transport to sustain neuronal viability, the hippocampus was analysed for inflammatory markers to

investigate hypoxia-driven neuroinflammation.

Expression levels of Iba-1, GFAP and IL-1 β were quantified to prove microglia and astrocyte activation as well as pro-inflammatory cytokine production (Cytokine networks in neuroinflammation | Nature Reviews Immunology [Internet]. [cited, 2025], respectively. Probiotic supplementation did not alter levels of these markers in the normoxic groups (NP and NC), suggesting no baseline effect on hippocampal inflammatory status. In contrast, under oxygen deprivation mice exhibited a pronounced state of neuroinflammation documented by the upregulation of all three markers in the hypoxia control group compared to NC (Fig. 6, panel A). Importantly, mice receiving probiotics under hypoxic conditions (HP) exhibited markedly reduced expression of Iba-1, GFAP, and IL-1 β compared to hypoxic controls, demonstrating the potentiality to preserve hippocampal homeostasis by modulating the inflammatory response triggered by oxygen deprivation. In line with these results, hypoxia also strongly upregulated iNOS expression (Fig. 6, panel A), denoting an increased production of nitric oxide, a reactive molecule produced only by activated macrophages and microglia in response to infection, inflammation or elevated oxidative stress (Inducible nitric oxide synthase gene expression in the brain during systemic inflammation | Nature Medicine [Internet]. [cited, 2025]). Importantly, probiotic treatment under hypoxic conditions significantly attenuated iNOS expression, further supporting the anti-inflammatory and

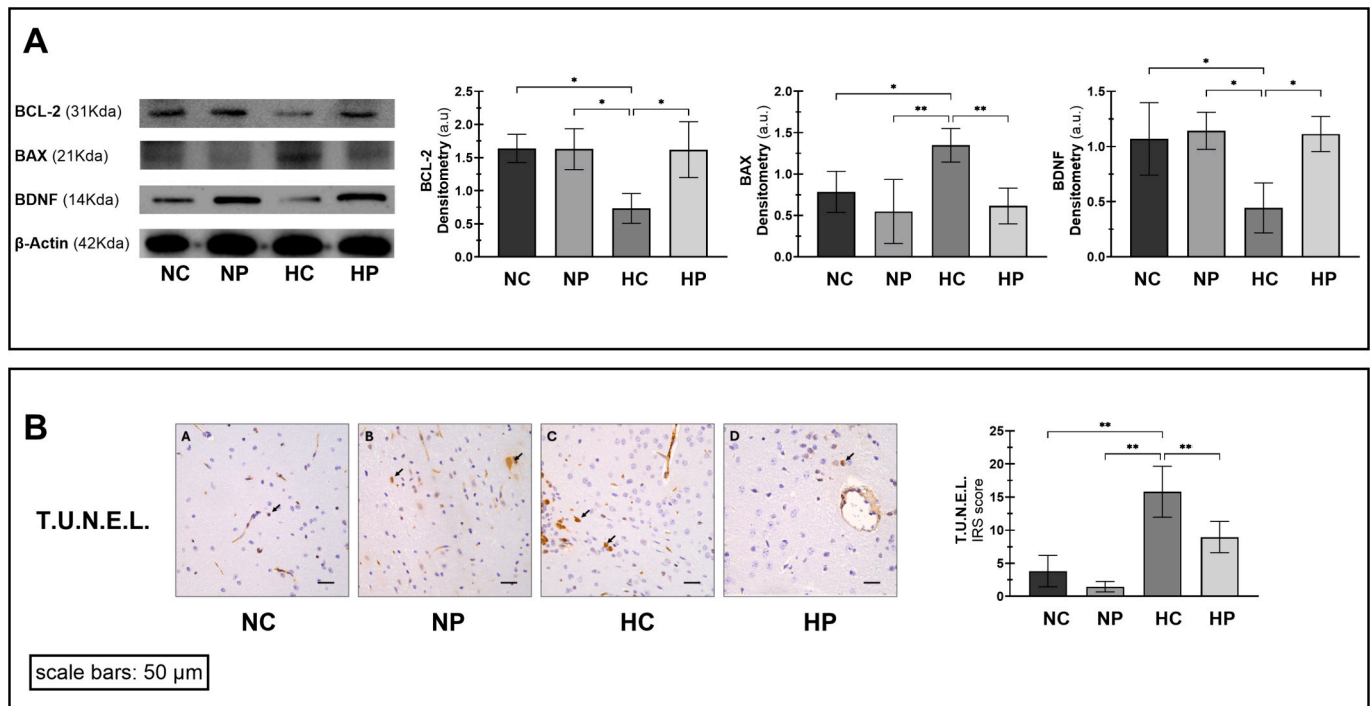


Fig. 7. Probiotic Treatment Modulates Apoptotic and Neurotrophic Signalling in the Hippocampus Under Hypoxia. Panel A: Western-blot analysis of hippocampal tissue for Bax, Bcl-2 and BDNF, the densitometry from three separate blots and a representative immunoblot are. Equal protein loading was verified by using an anti- β -actin antibody. Panel B: Representative TUNEL immunostaining for hippocampal section (scale bars: 50 μ m), quantified as immunoreactivity score (IRS) and expressed as mean $\pm$ SD. Data points marked with a single asterisk (*) are statistically significant ($p < 0.05$), while double asterisks (**) indicate $p < 0.001$, as determined by one-way ANOVA followed by Tukey's post hoc test.

neuroprotective potential in counteracting hypoxia-induced neuroinflammation.

These findings from WB analysis were corroborated by immunohistochemistry of brain tissue (Fig. 6, panel B), with Iba-1 and GFAP positive cells increased in the hypoxia control group. Iba-1 and GFAP expression levels were significantly lower in the hypoxic probiotic group. Together, these complementary approaches reinforce the evidence that probiotics mitigated hypoxia-induced neuroinflammation, thereby contributing to the maintenance of hippocampal homeostasis and modulation of the neuroimmune response under stress conditions.

3.4. Probiotic supplementation mitigates neuronal loss

To further evaluate the neuroprotective effects of the probiotic formulation on the hippocampus, the expression of apoptosis markers was examined by WB and TUNEL assay. The Bax/Bcl-2 ratio is a widely used indicator of apoptotic activation (Pepper et al., 1997), reflecting the balance between cell death and survival mechanisms. Levels of both Bax and Bcl-2 (Fig. 7, panel A) were not statistically different between the two normoxic groups, confirming the absence of a baseline effect. In contrast, hypoxic exposure induced a significant increase in Bax expression, accompanied by a decrease in Bcl-2. These molecular changes are indicative of enhanced susceptibility of hippocampal neurons to apoptosis under oxygen deprivation.

Remarkably, probiotic supplementation under hypoxia normalized both Bax and Bcl-2 expression to levels analogous to those observed in normoxic controls, (Fig. 7, panel A). This effect strongly suggests that probiotics can interfere with the mitochondrial apoptotic pathway, restoring the balance between pro- and anti-apoptotic signals, possibly reducing the processes leading to neuronal loss.

Activation of the hypoxic cascade was also assessed via the TUNEL assay, a widely utilized technique for detecting DNA fragmentation, a hallmark of late-stage apoptosis, that facilitates visualization and quantification of apoptotic cells in tissue sections. Consistent with

Western blot analyses, TUNEL staining of hippocampal tissue demonstrated a hypoxia-induced elevation in cells reporting DNA fragmentation, which was partially attenuated by probiotic intervention (Fig. 7, panel B). Neuroprotective effects are furthermore supported by the restoration of physiological levels of the neurotrophic factor BDNF under hypoxic conditions (Fig. 7, panel A).

3.5. Normalization of circulating neuroprotective hormone profiles following probiotic treatment

Effects of probiotic supplementation can be mediated by a group of neuroprotective hormones produced in the gastrointestinal tract. Hormones like GLP-1, GIP, ghrelin and leptin have a deep impact on general energy homeostasis (Obradovic et al., 2021; Baggio and Drucker, 2007; Kirchner et al., 2012), regulating appetite and energy uptake-expenditure ratio. They act as key metabolic regulators, slowing gastric emptying, stimulating insulin secretion after meals and signalling energy sufficiency to the brain. Only GIP resulted increased upon probiotic supplementation in mice under normoxia. In the hypoxic control group, circulating levels of GLP-1, GIP and leptin, were markedly reduced, suggesting a possible deleterious effect of oxygen restriction on energy metabolism and caloric uptake but interestingly they were partially restored in the group that received probiotics (Fig. 8, Panels A-D).

Notably, ghrelin was not found to be significantly influenced either by hypoxia or by the probiotic treatment. This finding may depend on the duration of hypoxic exposure, as studies on both humans and mice suggest that physiological adaptive responses tend to restore ghrelin levels after about 3 days of hypoxia exposure, thereby reducing the margin for probiotics to exert beneficial effects (Debevec, 2017). These findings highlight a potential mechanistic link between gut-derived hormonal signalling and the neuroprotective actions of probiotics under hypoxic stress.

Given that hypoxia concurrently altered circulating gut-derived

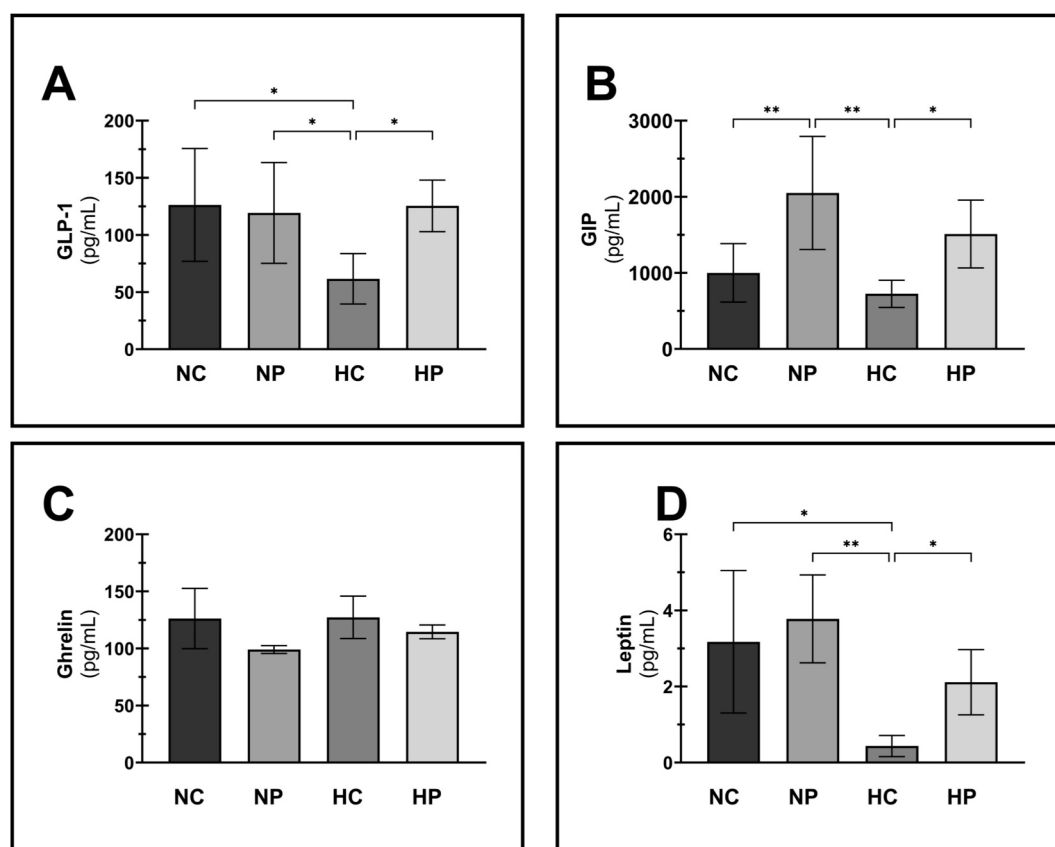


Fig. 8. Hypoxia impairs circulating gut hormones profile, which is restored upon probiotic administration. Panel A, B, C, D (respectively): GLP-1, GIP, ghrelin and leptin were determined in the plasma. Data points marked with a single asterisk (*) are statistically significant ($p < 0.05$), while double asterisks (**) indicate $p < 0.001$, as determined by one-way ANOVA followed by Tukey's post hoc test.

neuroprotective hormones and exacerbated neuronal loss and neuro-inflammation, we hypothesized that these peripheral perturbations could propagate to the cerebrovascular interface and compromise blood–brain barrier (BBB) integrity. Western blot analysis of brain homogenates confirmed that HC mice exhibited marked downregulation of the tight junction proteins zonulin-1, occludin, and claudin relative to normoxic controls, indicating hypoxia-driven BBB disruption. Conversely, probiotic administration restored the expression of these proteins in HP mice, suggesting that the intervention preserves cerebrovascular barrier integrity (Fig. 9).

These central findings prompted us to examine whether this neuro-protective effect was mirrored by restored intestinal barrier function and microbial homeostasis; accordingly, we next assessed intestinal tight junction proteins, gut microbiota composition, and circulating SCFA levels.

3.6. Preservation of gut barrier integrity via tight junction reinforcement under hypoxic stress

Hypoxia-induced gut dysbiosis may lead to perturbations in the intestinal barrier, that favours the entrance of deleterious substances in the systemic circulation, ultimately increasing inflammation (Marcari et al., 2025; *Altered gut microbiome in a mouse model of Gulf War Illness causes neuroinflammation and intestinal injury via leaky gut and TLR4 activation* | PLOS One [Internet]. [cited, 2025]). To assess gut barrier functionality, expression levels of tight junction proteins as zonulin-1 (ZO-1), claudin and occludin were examined by immunohistochemistry (Fig.10, panel A) and western blot (Fig.10, panel B) in the ileum, as a major site where microbial products, bile acids, and nutrients interact with the mucosa. Total expression levels of such proteins, as

well as the immunohistochemical reactivity is a common marker of gut-barrier functionality (Górecka et al., 2024). Hypoxia triggered a significant downregulation of all the markers assessed, suggesting gut-barrier disruption. Interestingly, this decrease was mitigated in the hypoxic probiotic group, suggesting that gut-barrier impairments may at least be attributable to changes in gut microbiota and that are counteracted by chronic probiotic supplementation able to prevent dysbiosis.

3.7. Probiotic modulation of gut microbiota and increased short-chain fatty acid production during hypoxia

Regarding microbial homeostasis, no significant differences in α -diversity were detected (Shannon index). PCoA based on Bray Curtis indicated differences in β -diversity (Fig.11, Panel A), suggesting distinct overall bacterial community structures and supporting a treatment-dependent shift in gut microbiota.

Functional metagenomes, predicted from 16s rDNA reads using PICRUST2 (Table 1), indicated potential enrichment of pathways for nucleic acid processing, glycolysis, nucleotide, carbohydrate, and fatty acid/lipid biosynthesis, suggesting that the microbial community shifted toward a more metabolically active and proliferative state (Table 1) in probiotic-treated hypoxic mice.

The predicted functional shifts are consistent with a microbiota that is more geared toward growth and active metabolism, potentially increasing production of microbial metabolites that can influence host epithelial and immune responses, as confirmed through ELISA detection of total SCFAs plasma levels, which were reduced under chronic hypoxia (HC) and restored by probiotics supplementation (HP) (Fig. 11 panel B). Among the top ASVs, several saccharolytic and SCFAs associated taxa (mainly within the families Ruminococcaceae, Lachnospiraceae,

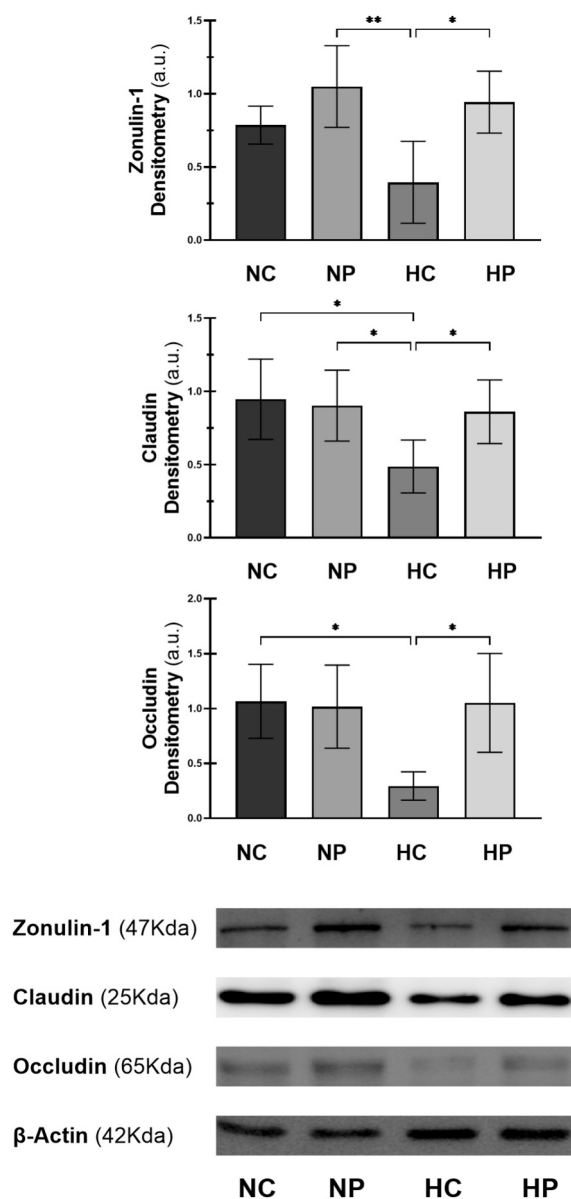


Fig. 9. Cerebral expression of tight junction proteins. Western-blot analysis of cerebral zonulin-1, claudin and occludin. The densitometry from three separate blots and a representative immunoblot are reported. Equal protein loading was verified by using an anti- β -actin antibody. Data points marked with a single asterisk (*) are statistically significant ($p < 0.05$), while double asterisks (**) indicate $p < 0.001$, as determined by one-way ANOVA followed by Tukey's post hoc test.

Muribaculaceae and Christensenellaceae) were reduced in hypoxic controls, consistent with a hypoxia induced loss of key fermentative bacteria (Supplementary table 1).

Plasma SCFAs concentrations and the relative abundance of bacterial species were correlated using Kendall's rank correlation coefficient.

Interestingly, the heatmap reveals that the hypoxic control group (HC) is characterized by a pattern of significant negative correlations involving several taxa associated with fermentative metabolism, including *Anaerostipes butyricus*, *Parabacteroides distasonis*, *Alistipes putredinis*, *Ruminococcus faecis*, and multiple members of the *Lachnospirillum* genus. Although these findings may initially appear counterintuitive, plasma SCFAs concentrations do not exclusively reflect microbial fermentative activity; rather, they represent the net balance of microbial production, intestinal absorption, epithelial metabolism, hepatic processing, and systemic utilization. Therefore, the negative

correlation in HC mice may reflect disrupted functional coupling between the gut microbiota and host metabolism (Fig. 11, panel C).

The concomitant perturbations in epithelium observed in the HC group, suggests impaired enterocytic physiology which could possibly reflect in a failed translation of the abundance of fermentative taxa into increased systemic availability of SCFAs.

The attenuation of this decoupled pattern in the hypoxic probiotic (HP) group further supports the hypothesis that probiotic administration partially preserved the functional coupling between gut microbial communities and host metabolic pathways (Fig. 11, panel C).

4. Discussion

Chronic hypoxia has attracted increasing interest in the field of neurodegeneration (Pham et al., 2021) as, cerebral hypoperfusion, a marked decrease in cerebral blood flow, is a well-documented event in the early stages of neurodegenerative diseases and may precede the onset of clinical symptom (Brown and Thore, 2011; Wierenga et al., 2014; Early role of vascular dysregulation on late-onset Alzheimer's disease based on multifactorial data-driven analysis | Nature Communications [Internet]. [cited, 2025]).

Because chronic oxygen deprivation shares several features with neurodegenerative conditions, the gut-brain axis may represent a relevant mechanistic interface and a potential therapeutic target. Intestinal microbiota may influence brain health by multiple mechanisms, including modulation of neuroinflammation, secretion of bioactive molecules and regulation of both the blood-brain barrier and intestinal barrier integrity (Quigley, 2017; Full article: The Impact of Gut Microbiota Disorders on the Blood-Brain Barrier [Internet]. [cited, 2025; Mou et al., 2022]).

Probiotics with proven neuroprotective effects in an AD mouse model (Bonfili et al., 2017; Bonfili et al., 2017; Bonfili et al., 2022; Bonfili et al., 2018) also showed significant results in rescuing blood oxygenation levels in preterm infants (Baldassarre et al., 2023) and COVID-19 patients (Ceccarelli et al., 2021). A central advantage of this multistrain strategy is the cooperative interaction among consortium members, wherein the presence of specific strains fosters a favorable microenvironment that supports the growth and functional activity of others.

Building on these findings, the present study investigated the impact of this probiotic mixture in mice undergoing chronic hypoxic exposure. In total, 40 mice were divided into 4 groups, two of them kept under low oxygen concentrations (12%) and two served as controls in normoxic conditions. Within each environmental condition, one group received probiotic supplementation while the corresponding control group received water during a standard laboratory diet. This design allowed evaluation of the probiotics' consortium potential neuroprotective role against chronic hypoxia-induced alterations, exploiting the modulation of gut-brain axis, without separating strain-specific contributions or minimal effective dosing.

In the present study probiotic supplementation attenuated HIF-1 α accumulation, partially restored PHD2 levels, and increased proteasomal activity under hypoxic conditions. These findings suggest that the probiotic modulates the HIF-1 α pathway by preserving oxygen-sensing mechanisms and promoting HIF-1 α degradation. Consistently, circulating EPO and brain GLUT-1 levels, two canonical HIF-1 α transcriptional targets, were reduced in probiotic-treated hypoxic mice, indicating a more effective adaptation to low oxygen availability. Probiotic supplementation restored these levels. The observed upregulation of the HIF-1 α target gene GLUT1 at the protein level in probiotic treated mice provides functional evidence that the stabilized HIF-1 α is transcriptionally active. Moreover, data are in agreement with recovered plasma metabolic hormone levels and PICRUST predictive outcomes, indicating a shift of the microbial community toward a more metabolically active state, even though direct functional assays will be required to confirm this interpretation.

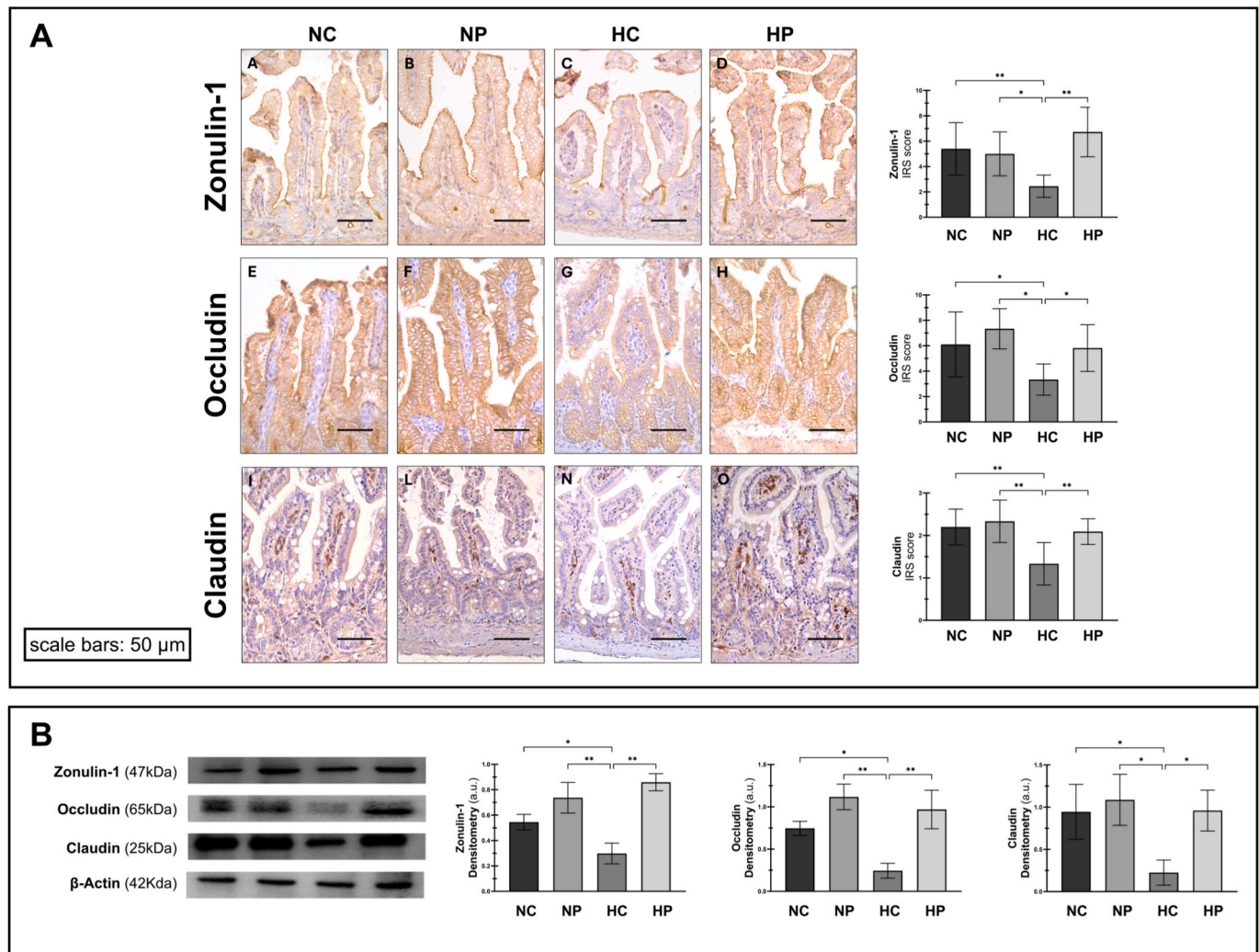


Fig. 10. Probiotic treatment prevents hypoxic decrease in tight junction proteins expression on mice ileum epithelial tissue. Panel A: Representative immunostaining for zonulin-1, occludin and claudin in ileum sections (scale bars: 50 μ m), quantified as immunoreactivity score (IRS) and expressed as mean $\pm$ SD. Panel B: Western-blot analysis of hippocampal tissue for zonulin-1, claudin and occludin, the densitometry from three separate blots and a representative immunoblot are reported. Equal protein loading was verified by using an anti- β -actin antibody. Data points marked with a single asterisk (*) are statistically significant ($p < 0.05$), while double asterisks (**) indicate $p < 0.001$, as determined by one-way ANOVA followed by Tukey's post hoc test.

HIF-1 α has also been implicated in neuroinflammatory responses associated with chronic hypoxia (Pham et al., 2021). In agreement with previous studies, hypoxic control mice displayed increased neuroinflammation, particularly within the hippocampus (Ma et al., 2024; Mechanisms underlying hypoxia-induced neuronal apoptosis, 2000; Zhu et al., 2023). Probiotic supplementation prevented the increase of the neuroinflammatory markers examined, possibly through the reduction of hypoxic stress and reduce HIF-1 α activation. Moreover, hypoxia-induced neuroinflammation has been associated with decreased BDNF expression (Zhu et al., 2026). Accordingly, cerebral BDNF expression was downregulated in the hypoxic control group, whereas probiotics restored BDNF levels to values closer to normoxia condition, supporting a neuroprotective and neurotrophic effect. Previous studies have shown that the same probiotic formulation exerts neuroprotective effects in transgenic mouse models of Alzheimer's disease in line with the hypothesis that modulation of gut microbiota composition may influence inflammatory pathways involved in neurodegeneration (Bonfili et al., 2017; Bonfili et al., 2022; Bonfili et al., 2018).

Accordingly, reduced tight junction protein expression in brain homogenates from hypoxic mice indicates hypoxia-induced BBB disruption, while probiotic supplementation partially restored these levels, suggesting that the intervention protects cerebrovascular integrity

under low-oxygen conditions.

Similarly, hypoxia is associated with perturbations in the gut barrier integrity, with numerous studies reporting such changes in mouse models exposed to high-altitude hypoxia (Li et al., 2025; Cheng et al., 2024). Consistent with these observations, hypoxic control mice showed reduced expression of intestinal tight junction proteins, suggesting increased gut permeability. Such alterations may facilitate the translocation of luminal pro-inflammatory molecules into the systemic circulation, thereby contributing to peripheral and central inflammatory responses. Interestingly, probiotic supplementation partially prevented these changes, supporting a protective effect on gut barrier integrity and further reinforcing the involvement of the gut-brain axis in the response to hypoxic stress.

Microbiota reshaping following probiotic administration was confirmed by changes in β -diversity and predicted functional shifts toward a more metabolically active and functionally balanced gut microbiota. The predicted increase in pathways related to nucleic acid biosynthesis, as well as carbohydrate, glycan, and cell structure biosynthesis, suggests that the probiotic-treated hypoxic group hosts a gut microbiota characterized by enhanced metabolic activity and enhanced bacterial growth. Moreover, the enrichment of fermentation pathways and the increased biosynthesis of amines, polyamines, and

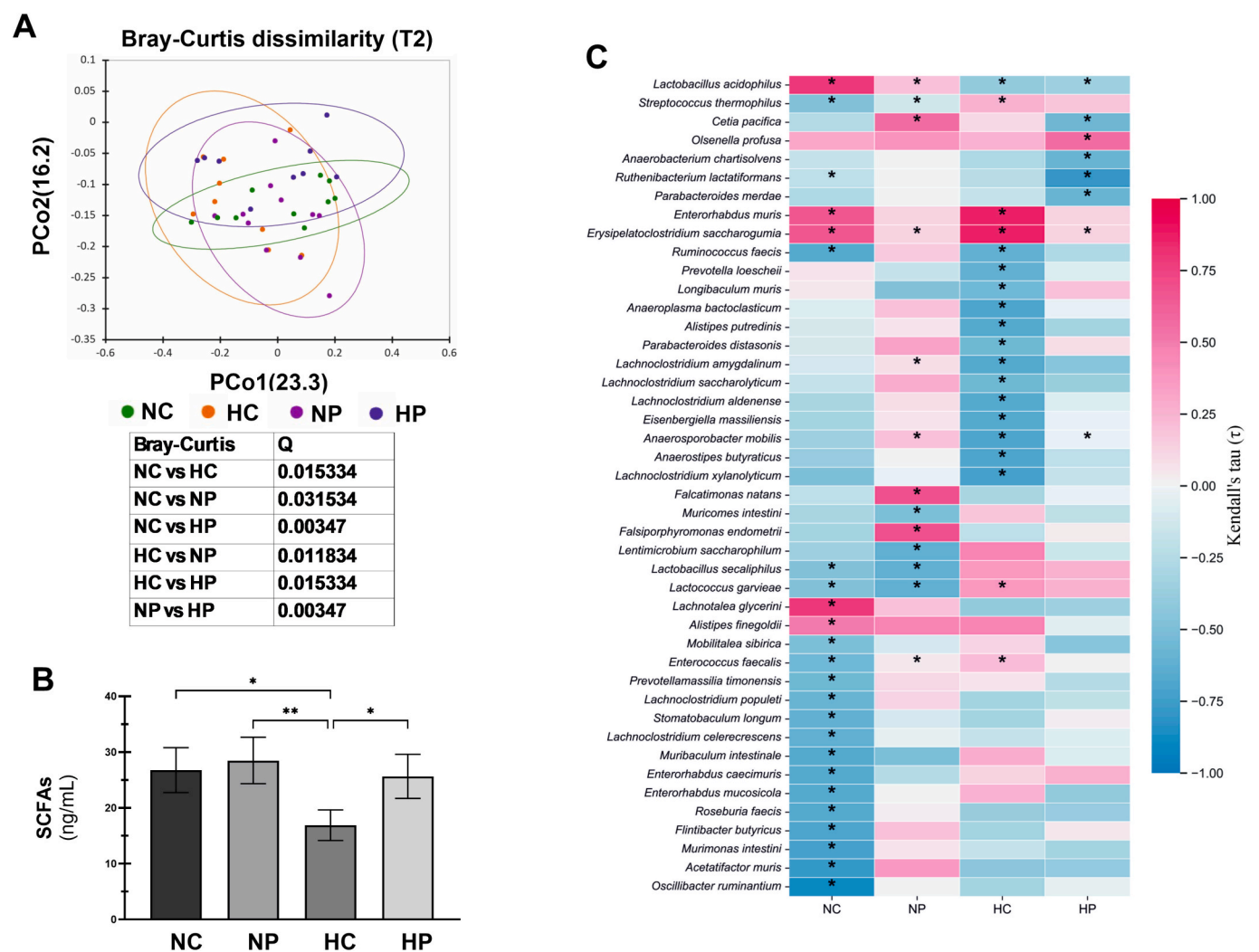


Fig. 11. Probiotic intervention restores hypoxic microbiota balance and potentiates circulating SCFA pools. Panel A: PCoA plot of bacterial β -diversity based on Bray-Curtis dissimilarity according to the different conditions tested. For each group, the 95% confidence interval has been drawn. Numbers between parenthesis represents the percentage of the total variance explained by the principal coordinates. Panel B. Plasma SCFAs concentrations quantified using ELISA. Data points marked with a single asterisk (*) are statistically significant ($p < 0.05$), while double asterisks (**) indicate $p < 0.001$, as determined by one-way ANOVA followed by Tukey's post hoc test. Panel C. Heatmap of correlations between relative species abundance and SCFAs (ng/mL) across the four sample groups (NC, NP, HC, HP). Color values represent Kendall's tau (τ) correlation coefficients. Only species exhibiting a relevant correlation ($|\tau| \geq 0.5$) in at least one of the analyzed groups are included in the map. Asterisks denote statistically significant correlations at alpha level 0.05.

aromatic compounds indicate a potential increase in the production of bioactive metabolites. Fermentation pathways are highly relevant for the production of short-chain fatty acids (SCFAs) that can cross the intestinal barrier and enter the systemic circulation, where they can act as signalling molecules contributing to energy metabolism and the regulation of key neurotransmitter pathways (Hu et al., 2018; Bacteria, 2026). Circulating SCFA levels are frequently reduced during gut dysbiosis, where beneficial microbial species, known producers of SCFAs (Frontiers | Vancomycin-induced gut microbiota dysbiosis aggravates allergic rhinitis in mice by altered short-chain fatty acids [Internet]. [cited, 2026; Zhan et al., 2023]) are decreased. In several murine models of chronic hypoxia, circulating SCFAs levels are also reduced, possibly because of hypoxia-induced alterations in gut microbiota composition and diversity (Zhang et al., 2022; Lu et al., 2025). However, the present study reveals a more complex relationship. In hypoxic control mice, Kendall's correlation analysis uncovered negative associations between plasma SCFAs and several recognized fermentative taxa, including *Anaerostipes butyraticus*, *Parabacteroides distasonis*, and *Lachnospirillum* species. This apparent paradox highlights that systemic SCFA availability reflects the net balance of microbial synthesis, intestinal

absorption, epithelial metabolism, and peripheral utilization—not merely bacterial production capacity. Thus, the inverse correlations likely indicate impaired enterocytic handling and barrier dysfunction in HC mice, which uncouple microbial abundance from systemic metabolite delivery. The attenuation of this pattern in HP mice suggests that probiotics re-established functional synchrony between the gut microbiota and host absorptive machinery, a concept further supported by the concurrent restoration of intestinal tight junction integrity.

Altogether, these findings indicate that chronic hypoxia activates neurodegeneration-like pathways in mice, including HIF-1 α -driven neuroinflammation, gut-barrier disruption, dysbiosis, and altered gut-hormone and SCFA profiles, which were partially counteracted by probiotic administration. The results suggest that intestinal microbiota and gut-brain axis modulation may play a key role in adaptation to hypoxic insult and offer a potential therapeutic strategy against neuroinflammation and cognitive decline triggered by oxygen-depriving conditions.

Future work should include metatranscriptomics or shotgun metagenomics, direct BBB permeability assays, long-term memory paradigms, and sex-stratified designs to confirm active microbial pathways,

Table 1

Functional metagenomes predicted from 16 s rDNA reads using PICRUST2 in hypoxic mice.

Predicted Function	HC (mean)	HP (mean)	p	q
Amine-and-Polyamine-Biosynthesis	5583.304 ± 2095.688	5798.234 ± 2249.629	0.002	0.034
Aminoacyl-tRNA-Charging	34578.956 ± 2951.472	40792.710 ± 7551.330	0.006	0.034
Cell-Structure-Biosynthesis	210488.364 ± 21146.577	253564.893 ± 44767.141	0.004	0.034
Fermentation	215714.302 ± 26095.680	261117.858 ± 54957.863	0.004	0.034
Glycan-Biosynthesis	30367.448 ± 3535.997	35046.448 ± 11093.367	0.006	0.034
Glycolysis	111089.538 ± 9797.725	129564.722 ± 22164.914	0.005	0.034
Nucleic-Acid-Processing	27516.876 ± 2442.431	30666.750 ± 4039.147	0.004	0.034
Nucleoside-and-Nucleotide-Biosynthesis	790033.176 ± 75970.321	949692.412 ± 132955.299	0.007	0.034
Nucleoside-and-Nucleotide-Degradation	104515.124 ± 16724.627	120295.231 ± 36069.700	0.004	0.034
Pentose-Phosphate-Pathways	60597.630 ± 5778.409	73437.621 ± 15922.531	0.008	0.034
Secondary-Metabolite-Biosynthesis	138750.762 ± 11879.973	164682.696 ± 24913.127	0.005	0.034
Superpathway-of-chorismate-metabolism	0.000 ± 0.000	832.812 ± 311.117	0.005	0.034
Aromatic-Compound-Biosynthesis	63537.260 ± 6392.322	77180.348 ± 19659.627	0.010	0.036
Carbohydrate-Biosynthesis	210953.262 ± 14294.601	252551.166 ± 42987.053	0.009	0.036
Fatty-Acid-and-Lipid-Biosynthesis	292262.420 ± 29791.967	379761.257 ± 71151.639	0.013	0.045

peripheral–central inflammatory transmission, behavioral persistence, and explore sex–dependent effects in probiotic neuroprotection.

CRediT authorship contribution statement

Francesco Maria Grasselli: Writing – original draft, Investigation. **Laura Bonfili:** Writing – review & editing, Methodology, Data curation. **Massimiliano Cuccioloni:** Writing – review & editing, Validation, Data curation. **Valentina Cekarini:** Writing – review & editing, Visualization, Validation. **Mauro Angeletti:** Writing – review & editing, Software, Data curation. **Massimiliano Marazzato:** Investigation, Formal analysis, Data curation. **Gregorio Sonsini:** Investigation, Data curation. **Massimo Ubaldi:** Validation, Investigation. **Lucia Biagini:** Visualization, Investigation. **Livio Galosi:** Investigation. **Giacomo Rossi:** Validation, Investigation, Data curation. **Anna Maria Eleuteri:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval and consent to participate

All experimental procedures were approved and authorized by the Italian Ministry of Health (Authorization n° 394/2024 – PR), following the national legislation (Legislative Decree 26/2014) and the European Communities Council Directives (2010/63/EU).

Consent for publication

Not applicable

Availability of data and materials

Raw sequencing data are available in the NCBI Sequence Read Archive under BioProject accession PRJNA1467165.

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbi.2026.106920>.

Data availability

Data will be made available on request.

References

A red lentils-based synbiotic cookie exerts neuroprotective effects in a mouse model of Alzheimer’s disease. *J Nutr Biochem*. Elsevier; 2025;141:109904. doi: 10.1016/j.jnutbio.2025.109904.

Acute exposure to simulated high-altitude hypoxia alters gut microbiota in mice | Archives of Microbiology [Internet]. [cited 2025 Sept 24]. <https://link.springer.com/article/10.1007/S00203-022-03031-4>. Accessed 24 Sept 2025.

Altered gut microbiome in a mouse model of Gulf War Illness causes neuroinflammation and intestinal injury via leaky gut and TLR4 activation | PLOS One [Internet]. [cited 2025 Sept 25]. <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0172914>. Accessed 25 Sept 2025.

Antunes, M., Biala, G., 2012. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn. Process.* 13, 93–110. <https://doi.org/10.1007/s10339-011-0430-z>.

Gut Bacteria and Neurotransmitters [Internet]. [cited 2026 Mar 27]. <https://www.mdpi.com/2076-2607/10/9/1838>. Accessed 27 Mar 2026.

Baggio, L.L., Drucker, D.J., 2007. Biology of incretins: GLP-1 and GIP. *Gastroenterology* 132, 2131–2157. <https://doi.org/10.1053/j.gastro.2007.03.054>.

Baldassarre, M.E., Marazzato, M., Pensa, M., Loverro, M.T., Quercia, M., Lombardi, F., et al., 2023. SLAB51 Multi-Strain Probiotic Formula Increases Oxygenation in Oxygen-Treated Preterm Infants. *Nutrients* 15, 3685. <https://doi.org/10.3390/nu15173685>.

Bonfili, L., Cekarini, V., Berardi, S., Scarpona, S., Suchodolski, J.S., Nasuti, C., et al., 2017. Microbiota modulation counteracts Alzheimer’s disease progression influencing neuronal proteolysis and gut hormones plasma levels. *Sci. Rep.* 7, 2426. <https://doi.org/10.1038/s41598-017-02587-2>.

Bonfili, L., Cekarini, V., Cuccioloni, M., Angeletti, M., Berardi, S., Scarpona, S., et al., 2018. SLAB51 Probiotic Formulation Activates SIRT1 Pathway Promoting Antioxidant and Neuroprotective Effects in an AD Mouse Model. *Mol. Neurobiol.* 55, 7987–8000. <https://doi.org/10.1007/s12035-018-0973-4>.

Bonfili, L., Gong, C., Lombardi, F., Cifone, M.G., Eleuteri, A.M., 2022. Strategic Modification of Gut Microbiota through Oral Bacteriotherapy Influences Hypoxia Inducible Factor-1α: Therapeutic Implication in Alzheimer’s Disease. *Int J Mol Sci. Multidisciplinary Digital Publishing Institute* 23, 357. <https://doi.org/10.3390/ijms23010357>.

Bonfili, L., Grasselli, F.M., Cuccioloni, M., Cekarini, V., Lufano, D., Vittadini, E., et al., 2025. A red lentils-based synbiotic cookie exerts neuroprotective effects in a mouse model of Alzheimer’s disease. *J. Nutr. Biochem.* 141, 109904. <https://doi.org/10.1016/j.jnutbio.2025.109904>.

- Brown, W.R., Thore, C.R., 2011. Review: Cerebral microvascular pathology in ageing and neurodegeneration. *Neuropathol. Appl. Neurobiol.* 37, 56–74. <https://doi.org/10.1111/j.1365-2990.2010.01139.x>.
- Burtscher, M., Millet, G.P., Burtscher, J., 2022. Hypoxia Conditioning for High-Altitude Pre-acclimatization. *J Sci Sport Exerc.* 4, 331–345. <https://doi.org/10.1007/s42978-021-00150-0>.
- Ceccarelli, G., Marazzato, M., Celani, L., Lombardi, F., Piccirilli, A., Mancone, M., et al., 2021. Oxygen Sparing Effect of Bacteriotherapy in COVID-19. *Nutrients*. Multidisciplinary Digital Publishing Institute 13, 2898. <https://doi.org/10.3390/nu13082898>.
- Cellular adaptation to hypoxia through hypoxia inducible factors and beyond | *Nature Reviews Molecular Cell Biology* [Internet]. [cited 2025 Sept 25]. <https://www.nature.com/articles/s41580-020-0227-y>. Accessed 25 Sept 2025.
- Cheng, J., Sun, Y., Zhao, Y., Guo, Q., Wang, Z., Wang, R., 2024. Research Progress on the Mechanism of Intestinal Barrier Damage and Drug Therapy in a High Altitude Environment. *Curr Drug Deliv. Bentham Science Publishers* 21, 807–816. <https://doi.org/10.2174/1567201820666230309090241>.
- Cytokine networks in neuroinflammation | *Nature Reviews Immunology* [Internet]. [cited 2025 Sept 25]. <https://www.nature.com/articles/nri.2016.123>. Accessed 25 Sept 2025.
- DADA2: High-resolution sample inference from Illumina amplicon data | *Nature Methods* [Internet]. [cited 2026 Jan 2]. <https://www.nature.com/articles/nmeth.3869>. Accessed 2 Jan 2026.
- de Gooyer, J.M., Versleijen-Jonkers, Y.M.H., Hillebrandt-Roefien, M.H.S., Frielink, C., Desar, I.M.E., de Wilt, J.H.W., et al., 2020. Immunohistochemical selection of biomarkers for tumor-targeted image-guided surgery of myxofibrosarcoma. *Sci Rep. Nat. Publ. Group* 10, 2915. <https://doi.org/10.1038/s41598-020-59735-4>.
- Debevec, T., 2017. Hypoxia-Related Hormonal Appetite Modulation in Humans during Rest and Exercise: Mini Review. *Front Physiol* [Internet]. *Frontiers*. <https://doi.org/10.3389/fphys.2017.00366> [cited 2026 Jan 2];8.
- Della Rocca, Y., Fonticoli, L., Rajan, T.S., Trubiani, O., Caputi, S., Diomedea, F., et al., 2022. Hypoxia: molecular pathophysiological mechanisms in human diseases. *J. Physiol. Biochem.* 78, 739–752. <https://doi.org/10.1007/s13105-022-00912-6>.
- Douglas, G.M., Maffei, V.J., Zaneveld, J.R., Yurgel, S.N., Brown, J.R., Taylor, C.M., et al., 2020. PICRUST2 for prediction of metagenome functions. *Nat Biotechnol. Nature Publishing Group* 38, 685–688. <https://doi.org/10.1038/s41587-020-0548-6>.
- Early role of vascular dysregulation on late-onset Alzheimer's disease based on multifactorial data-driven analysis | *Nature Communications* [Internet]. [cited 2025 Sept 25]. <https://www.nature.com/articles/ncomms11934>. Accessed 25 Sept 2025.
- Frontiers | Vancomycin-induced gut microbiota dysbiosis aggravates allergic rhinitis in mice by altered short-chain fatty acids [Internet]. [cited 2026 Mar 27]. <https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2022.1002084/full>. Accessed 27 Mar 2026.
- Full article: The Impact of Gut Microbiota Disorders on the Blood-Brain Barrier [Internet]. [cited 2025 Sept 25]. <https://www.tandfonline.com/doi/full/10.2147/IDR.S254403>. Accessed 25 Sept 2025.
- Górecka, A., Jura-Pótorak, A., Kozma, E.M., Szeremeta, A., Olczyk, K., Komosińska-Vascek, K., 2024. Biochemical Modulators of Tight Junctions (TJs): Occludin, Claudin-2 and Zonulin as Biomarkers of Intestinal Barrier Leakage in the Diagnosis and Assessment of Inflammatory Bowel Disease Progression. *Molecules*. Multidisciplinary Digital Publishing Institute 29, 4577. <https://doi.org/10.3390/molecules29194577>.
- Han, N., Pan, Z., Liu, G., Yang, R., Hypoxia, Y.B., 2021. The “Invisible Pusher” of Gut Microbiota. *Front Microbiol* [Internet]. *Frontiers*. <https://doi.org/10.3389/fmicb.2021.690600> [cited 2025 Sept 24];12.
- Hu, J., Lin, S., Zheng, B., Cheung, P.C.K., 2018. Short-chain fatty acids in control of energy metabolism. *Crit Rev Food Sci Nutr. Taylor & Francis* 58, 1243–1249. <https://doi.org/10.1080/10408398.2016.1245650>.
- Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1 α is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci. Proceedings of the National Academy of Sciences*; 1998;95:7987–92. doi: 10.1073/pnas.95.14.7987.
- Inducible nitric oxide synthase gene expression in the brain during systemic inflammation | *Nature Medicine* [Internet]. [cited 2025 Sept 25]. <https://www.nature.com/articles/nm0596-581>. Accessed 25 Sept 2025.
- Kim, S.-K., Guevarra, R.B., Kim, Y.-T., Kwon, J., Kim, H., Cho, J.H., et al., 2019. Role of Probiotics in Human Gut Microbiome-Associated Diseases. *J. Microbiol. Biotechnol.* 29, 1335–1340. <https://doi.org/10.4014/jmb.1906.06064>.
- Kirchner H, Heppner KM, Tschöp MH. The Role of Ghrelin in the Control of Energy Balance. In: Joost H-G, editor. *Appetite Control* [Internet]. Berlin, Heidelberg: Springer; 2012 [cited 2025 Sept 25]. p. 161–84. doi: 10.1007/978-3-642-24716-3_7.
- Kumari M, Yadav A, Kumari A, Rai C, Varshney R, Meena RC. High-Altitude-Induced Dysbiosis in Gut and Oral Microbiome: Strategies for Restoration of Microbiota. In: Srivastava S, Sharma M, Varshney R, editors. *Health Sustainance Extreme Environ Mil Perspect* [Internet]. Singapore: Springer Nature; 2025 [cited 2025 Sept 24]. p. 99–113. doi: 10.1007/978-981-96-8642-1_6.
- Lestón Pinilla, L., Ugun-Klusek, A., Rutella, S., De Girolamo, L.A., 2021. Hypoxia Signaling in Parkinson's Disease: There Is Use in Asking “What HIF?” *Biology*. Multidisciplinary Digital Publishing Institute 10, 723. <https://doi.org/10.3390/biology10080723>.
- Li, W., Wang, Y., Shi, Y., He, F., Zhao, Z., Liu, J., et al., 2025. The gut microbiota mediates memory impairment under high-altitude hypoxia via the gut-brain axis in mice. *FEBS J. John Wiley & Sons, Ltd* 292, 809–826. <https://doi.org/10.1111/febs.17365>.
- Lu, G., Rili, G., Shuang, M., 2025. Impact of hypoxia on the hippocampus: A review. *Medicine (Baltimore)* 104, e41479. <https://doi.org/10.1097/MD.00000000000041479>.
- Lu, M.-L., Wu, J.-L., Zhu, J.-W., Liu, L., Li, M.-Z., Yu, Y., et al., 2025. Changes in the gut microbiota in mice exposed to chronic intermittent hypoxia. *J. Med. Microbiol.* 74, 002069. <https://doi.org/10.1099/jmm.0.002069>.
- Ma, Y.-Y., Li, X., Yu, Z.-Y., Luo, T., Tan, C.-R., Bai, Y.-D., et al., 2024. Oral antioxidant edaravone protects against cognitive deficits induced by chronic hypobaric hypoxia at high altitudes. *Transl Psychiatry. Nat. Publ. Group* 14, 415. <https://doi.org/10.1038/s41398-024-03133-1>.
- Marazzato, M., Zicari, A.M., Aleandri, M., Conte, A.L., Longhi, C., Vitanza, L., et al., 2020. 16S Metagenomics Reveals Dysbiosis of Nasal Core Microbiota in Children With Chronic Nasal Inflammation: Role of Adenoid Hypertrophy and Allergic Rhinitis. *Front Cell Infect Microbiol* [Internet]. *Frontiers*. <https://doi.org/10.3389/fcimb.2020.00458> [cited 2026 Jan 2];10.
- Marcari, A.B., Paiva, A.D., Simon, C.R., Dos Santos, M.E.S.M., 2025. Leaky Gut Syndrome: An Interplay Between Nutrients and Dysbiosis. *Curr. Nutr. Rep.* 14, 25. <https://doi.org/10.1007/s13668-025-00614-7>.
- Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal.* 17, 10–12. <https://doi.org/10.14806/ej.17.1.200>.
- McNaught KSTP, Jenner P, 2001. Proteasomal function is impaired in substantia nigra in Parkinson's disease. *Neurosci. Lett.* 297, 191–194. [https://doi.org/10.1016/S0304-3940\(00\)01701-8](https://doi.org/10.1016/S0304-3940(00)01701-8).
- Mechanisms underlying hypoxia-induced neuronal apoptosis. *Prog Neurobiol. Pergamon*; 2000;62:215–49. doi: 10.1016/S0301-0082(00)00011-3.
- Mou, Y., Du, Y., Zhou, L., Yue, J., Hu, X., Liu, Y., et al., 2022. Gut Microbiota Interact With the Brain Through Systemic Chronic Inflammation: Implications on Neuroinflammation, Neurodegeneration, and Aging. *Front Immunol* [Internet]. *Frontiers*. <https://doi.org/10.3389/fimmu.2022.796288> [cited 2025 Sept 25];13.
- [45] NLRP3 deficiency protects against hypobaric hypoxia induced neuroinflammation and cognitive dysfunction. *Ecotoxicol Environ Saf. Academic Press*; 2023;255: 114828. doi: 10.1016/j.ecoenv.2023.114828.
- Obradovic, M., Sudar-Milovanovic, E., Soskic, S., Essack, M., Arya, S., Stewart, A.J., et al., 2021. Leptin and Obesity: Role and Clinical Implication. *Front. Endocrinol.* 12, 585887. <https://doi.org/10.3389/fendo.2021.585887>.
- Önder, Ö., 2025. Challenges of Cerebral Hemodynamic Disorders and Hypoxic-Ischemic Encephalopathy: Evaluating Current and Emerging Approaches from Neurological Emergencies to Intensive Care. *Cyprus J Med Sci* [Internet]. *Cyprus. J. Med. Sci.* <https://doi.org/10.4274/cjms.2025.2024-82> [cited 2025 Sept 24];.
- Pepper, C., Hoy, T., Bentley, D.P., 1997. Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical resistance. *Br J Cancer. Nature Publishing Group* 76, 935–938. <https://doi.org/10.1038/bjc.1997.487>.
- Pham K, Parikh K, Heinrich EC. Hypoxia and Inflammation: Insights From High-Altitude Physiology. *Front Physiol* [Internet]. *Frontiers*; 2021 [cited 2025 Sept 25];12. doi: 10.3389/fphys.2021.676782.
- Pham K, Parikh K, Heinrich EC. Hypoxia and Inflammation: Insights From High-Altitude Physiology. *Front Physiol* [Internet]. *Frontiers*; 2021 [cited 2025 Sept 25];12. doi: 10.3389/fphys.2021.676782.
- Prut, L., Belzung, C., 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur. J. Pharmacol.* 463, 3–33. [https://doi.org/10.1016/S0014-2999\(03\)01272-X](https://doi.org/10.1016/S0014-2999(03)01272-X).
- Quigley, E.M.M., 2017. Microbiota-Brain-Gut Axis and Neurodegenerative Diseases. *Curr. Neurol. Neurosci. Rep.* 17, 94. <https://doi.org/10.1007/s11910-017-0802-6>.
- Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2 | *Nature Biotechnology* [Internet]. [cited 2026 Jan 2]. <https://www.nature.com/articles/s41587-019-0209-9>. Accessed 2 Jan 2026.
- Ross, C.A., Poirier, M.A., 2004. Protein aggregation and neurodegenerative disease. *Nat Med. Nature Publishing Group* 10, S10–S17. <https://doi.org/10.1038/nm1066>.
- Salceda, S., Caro, J., 1997. Hypoxia-inducible Factor 1 α (HIF-1 α) Protein Is Rapidly Degraded by the Ubiquitin-Proteasome System under Normoxic Conditions: ITS STABILIZATION BY HYPOXIA DEPENDS ON REDOX-INDUCED CHANGES*. *J. Biol. Chem.* 272, 22642–22647. <https://doi.org/10.1074/jbc.272.36.22642>.
- Sarita, B., Samadhan, D., Hassan, M.Z., Kovaleva, E.G., 2025. A comprehensive review of probiotics and human health-current perspective and applications. *Front Microbiol* [Internet]. *Frontiers*. <https://doi.org/10.3389/fmicb.2024.1487641> [cited 2025 Sept 24];15.
- Saugy, J.J., Schmoutz, T., Botré, F., 2022. Altitude and Erythropoietin: Comparative Evaluation of Their Impact on Key Parameters of the Athlete Biological Passport: A Review. *Front Sports Act Living.* 4, 864532. <https://doi.org/10.3389/fspor.2022.864532>.
- Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods. Nature Publishing Group* 9, 671–675. <https://doi.org/10.1038/nmeth.2089>.
- Smith, N.K., Hackett, T.A., Galli, A., Flynn, C.R., 2019. GLP-1: Molecular mechanisms and outcomes of a complex signaling system. *Neurochem. Int.* 128, 94–105. <https://doi.org/10.1016/j.neuint.2019.04.010>.
- Thibaudau, T.A., Anderson, R.T., Smith, D.M., 2018. A common mechanism of proteasome impairment by neurodegenerative disease-associated oligomers. *Nat Commun. Nat. Publ. Group* 9, 1097. <https://doi.org/10.1038/s41467-018-0350-0>.
- Vizcardo-Galindo, G., León-Velarde, F., Villafuerte, F.C., 2020. High-Altitude Hypoxia Decreases Plasma Erythropoietin Soluble Receptor Concentration in Lowlanders. *High Alt. Med. Biol.* 21, 92–98. <https://doi.org/10.1089/ham.2019.0118>.

- Wierenga, C.E., Hays, C.C., Zlatar, Z.Z., 2014. Cerebral Blood Flow Measured by Arterial Spin Labeling MRI as a Preclinical Marker of Alzheimer's Disease. *J Alzheimer's Dis.* SAGE Publications 42, S411–S419. <https://doi.org/10.3233/JAD-141467>.
- Wolters FJ, Zonneveld HI, Hofman A, Lugt A van der, Koudstaal PJ, Vernooij MW, et al. Cerebral Perfusion and the Risk of Dementia. *Circulation* [Internet]. Lippincott Williams & Wilkins; Hagerstown, MD; 2017 [cited 2025 Sept 24]; doi: 10.1161/CIRCULATIONAHA.117.027448.
- Wright, J., Harwood, B., Burnet, S., 2021. Reliability and validity of the Hypoxico Everest Summit II altitude generator. *Proc Inst Mech Eng Part P J Sports. Eng. Technol.* 236. <https://doi.org/10.1177/1754337121995976>.
- Zhan, Y., Al-Nusaif, M., Ding, C., Zhao, L., Dong, C., 2023. The potential of the gut microbiome for identifying Alzheimer's disease diagnostic biomarkers and future therapies. *Front Neurosci* [Internet]. Frontiers. <https://doi.org/10.3389/fnins.2023.1130730> [cited 2026 Mar 31];17.
- Zhang, J.-Z., Behrooz, A., Ismail-Beigi, F., 1999. Regulation of glucose transport by hypoxia. *Am. J. Kidney Dis.* 34, 189–202. [https://doi.org/10.1016/S0272-6386\(99\)70131-9](https://doi.org/10.1016/S0272-6386(99)70131-9).
- Zhang, X., Le, W., 2010. Pathological role of hypoxia in Alzheimer's disease. *Exp. Neurol.* 223, 299–303. <https://doi.org/10.1016/j.expneurol.2009.07.033>.
- Zhang, Y., Luo, H., Niu, Y., Yang, X., Li, Z., Wang, K., et al., 2022. Chronic intermittent hypoxia induces gut microbial dysbiosis and infers metabolic dysfunction in mice. *Sleep Med.* 91, 84–92. <https://doi.org/10.1016/j.sleep.2022.02.003>.
- Zhao, Z., Cui, D., Wu, G., Ren, H., Zhu, X., Xie, W., et al., 2022. Disrupted gut microbiota aggravates working memory dysfunction induced by high-altitude exposure in mice. *Front Microbiol* [Internet]. Frontiers. <https://doi.org/10.3389/fmicb.2022.1054504> [cited 2025 Sept 24];13.
- Zhu, X., Liu, H., Wang, D., Guan, R., Zou, Y., Li, M., et al., 2023. NLRP3 deficiency protects against hypobaric hypoxia induced neuroinflammation and cognitive dysfunction. *Ecotoxicol. Environ. Saf.* 255, 114828. <https://doi.org/10.1016/j.ecoenv.2023.114828>.
- Zhu, M., Zhang, Z., Fu, J., Guo, J., Zhan, H., Guo, X., et al., 2026. Orexin A ameliorates high altitude induced memory retrieval impairment via BDNF/TrkB and PI3K/AKT/mTOR signaling pathways. *Behav. Brain Res.* 496, 115866. <https://doi.org/10.1016/j.bbr.2025.115866>.