

## Modulation of the Nuclear factor erythroid 2-related factor 2 (Nrf2) pathway by the probiotic *Limosilactobacillus reuteri* DSM 17938 prevents diet-induced rat brain dysfunction

Arianna Mazzoli<sup>a</sup>, Maria Stefania Spagnuolo<sup>b</sup>, Martina Nazzaro<sup>a</sup>, Angela Di Porzio<sup>a</sup>,  
Valentina Barrella<sup>a</sup>, Antonio Dario Troise<sup>b</sup>, Sabrina De Pascale<sup>b</sup>, Andrea Scaloni<sup>b</sup>,  
Gianluigi Mauriello<sup>c</sup>, Susanna Iossa<sup>a,d,e,\*</sup>, Luisa Cigliano<sup>a,e,\*</sup>

<sup>a</sup> Department of Biology, University of Naples Federico II, Complesso Universitario Monte S. Angelo, Edificio 7, Via Cintia, 80126 Naples, Italy

<sup>b</sup> Institute for the Animal Production System in the Mediterranean Environment, National Research Council, Piazzale Enrico Fermi 1, 80055 Portici, Italy

<sup>c</sup> Department of Agricultural Sciences, University of Naples Federico II, via Università 100, 80055 Portici, Italy

<sup>d</sup> NBFC, National Biodiversity Future Center, Palermo 90133, Italy

<sup>e</sup> Task Force on Microbiome Studies, University of Naples Federico II, Italy

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### ABSTRACT

Dietary patterns high in fat and sugar promote brain dysfunction, but the preventive efficacy of probiotics has been little explored. This study investigated whether the administration of *Limosilactobacillus reuteri* DSM 17938 (*L.reuteri*; 10<sup>8</sup> CFU/day) counteracts cognitive dysfunction and brain redox unbalance induced in male Wistar rats fed a western diet (WD) for 8 weeks.

The results showed that *L. reuteri* prevented the WD-associated impairment in brain memory function, as evaluated by NOR test, and consistently increased specific molecular markers of synaptic plasticity. The condition of oxidative stress induced by WD, namely enhanced oxidation to proteins and lipids, increased oxidants-producing enzymes as well as reduced nuclear Nrf2 and Nrf2-dependent antioxidant enzymes, was ameliorated by the probiotic. Unexpectedly, mitochondria displayed a diet-induced increase in oxidative capacity, not modified by the probiotic. A protective effect of the probiotic on gut absorption of fructose and its further delivery to brain was evidenced as mechanism involved in cortex redox homeostasis. These findings evidence the potential utility of *L. reuteri* against WD-induced brain dysfunction and implicate the Nrf2 pathway as a potential mechanism for this effect.

### 1. Introduction

The Western diet (WD), rich in saturated fats and added sugars, like sucrose or high-fructose corn syrup, markedly contributing to the increased intake of fructose, originated in western countries, but is now diffused also in eastern and developing countries (Imamura et al., 2015). Since this diet is responsible for the significant development of several metabolic diseases (Clemente-Suárez et al., 2023), unraveling its impact on health is of critical importance for the identification of new strategies

able to mitigate its damaging effects. In particular, WD promotes cognitive dysfunction (López-Taboada et al., 2020; Spagnuolo et al., 2020), even if the molecular mechanisms driving this impairment are not fully understood. In this context, one possibility is that WD consumption induces oxidative stress in the brain, which in turn is responsible for organ dysfunction and cognitive disturbances. Indeed, the brain is more sensitive to oxidative damage compared to other organs due to its higher levels of oxygen consumption and weaker antioxidant defenses (Cobley et al., 2018); moreover, the relationship

\* Corresponding authors at: Department of Biology, University of Naples Federico II, Complesso Universitario Monte S. Angelo, Edificio 7, Via Cintia I-80126 Napoli, Italy (L. Cigliano). Department of Biology, Complesso Universitario Monte S. Angelo, Edificio 7, Via Cintia - I-80126 Napoli, Italy (S. Iossa).

E-mail addresses: [arianna.mazzoli@unina.it](mailto:arianna.mazzoli@unina.it) (A. Mazzoli), [mariastefania.spagnuolo@cnr.it](mailto:mariastefania.spagnuolo@cnr.it) (M. Stefania Spagnuolo), [martina.nazzaro@unina.it](mailto:martina.nazzaro@unina.it) (M. Nazzaro), [angela.diporzio@unina.it](mailto:angela.diporzio@unina.it) (A. Di Porzio), [valentina.barrella@unina.it](mailto:valentina.barrella@unina.it) (V. Barrella), [antoniodario.troise@cnr.it](mailto:antoniodario.troise@cnr.it) (A. Dario Troise), [sabrina.depascale@ispaam.cnr.it](mailto:sabrina.depascale@ispaam.cnr.it) (S. De Pascale), [andrea.scaloni@cnr.it](mailto:andrea.scaloni@cnr.it) (A. Scaloni), [gianluigi.mauriello@unina.it](mailto:gianluigi.mauriello@unina.it) (G. Mauriello), [susanna.iossa@unina.it](mailto:susanna.iossa@unina.it) (S. Iossa), [luisa.cigliano@unina.it](mailto:luisa.cigliano@unina.it) (L. Cigliano).

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between oxidative stress and cognitive decline is well documented (Salim, 2017; Hajjar et al., 2018; Kandlur et al., 2020; Li et al., 2023).

An important role in the protection against brain oxidative damage is played by nuclear factor erythroid 2-related factor 2 (Nrf2) (Johnson et al., 2008), which, under redox stress conditions, interacts with the antioxidant response element, regulating phase II antioxidant responses (Cigliano et al., 2019; Heurtaux et al., 2022). Stimulation of Nrf2 might represent a way to ameliorate brain health; indeed, recent investigations focused on the study of natural products that can positively regulate the Nrf2 pathway to reduce oxidative damage to the nervous system (Moratilla-Rivera et al., 2023). As a matter of fact, Nrf2 activity can be modified by daily consumption of specific food and phytochemicals, such as specific polyphenols, curcumin, flavonoids (Moratilla-Rivera et al., 2023), but less is known on the potential impact of probiotics, despite huge growing data evidencing the importance of the gut-brain axis (Dinan and Cryan, 2017; Schächtle and Rosshart, 2021; Boehme et al., 2023) and pointing to the probiotic administration as a strategy to counteract diet-induced brain dysfunction (Ji et al., 2021; Jena et al., 2022; Lof et al., 2022). In this context, a recent study showed the antioxidant activities of novel peptides from *Limosilactobacillus reuteri* DSM 17938-fermented food (Tyagi et al., 2023), as well as the effect of the administration of this probiotic on the serum levels of serotonin and brain-derived neurotrophic factor (BDNF) in humans (Riezzo et al., 2019). In addition, we recently showed the beneficial impact of *L. reuteri* in counteracting WD-induced metabolic derangement in gut and liver (Abuqwider et al., 2023; Di Porzio et al., 2023). However, a comprehensive investigation on the potential efficacy of this probiotic strain in WD-induced cognitive dysfunction and redox alterations is still lacking.

Therefore, the main objective of this study was to investigate whether the intake of *L. reuteri* DSM 17938 can counteract cognitive dysfunction and brain redox imbalance induced in WD-fed male rats. In particular, we investigated whether behavioral changes elicited by WD and concomitant probiotic administration could be related to redox homeostasis, Nrf2-mediated antioxidants molecules and molecular markers of synaptic and memory function.

## 2. Materials and methods

### 2.1. Materials

Bovine serum albumin fraction V (BSA), non-fat milk, salts and buffers were purchased from DelTech (Naples, Italy). Fuji Super RX film, FujiFilm Man-X Developer, and FujiFilm Man-X Fixer were from Laboratorio Elettronico Di Precisione (Naples, Italy). Water, methanol and acetonitrile were of mass spectrometry grade and were obtained from Merck (Darmstadt, Germany). Along with derivatizing agents 3-nitrophenylhydrazine (3-NPH), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and quinic acid, all the analytical standards including lithium acetoacetate, sodium  $\beta$ -hydroxybutyrate, and internal standards  $^{13}\text{C}_2$ -acetic acid,  $^{13}\text{C}_3$ -propionic acid and  $^{13}\text{C}_4$ -butyric acid were purchased from Sigma-Merck (Darmstadt, Germany). Pyridine was obtained from Fisher Scientific (Bremen, Germany).

### 2.2. Cultivation of *L. Reuteri* DSM 17938

*L. reuteri* DSM 17938 was kindly provided by BioGaia (Noos S.r.l.; BioGaia AB, Stockholm, Sweden). It was cultured in MRS Broth (OXOID Ltd., Basingstoke, Hampshire, England) at 37 °C, checked for purity and maintained on MRS Agar (Oxoid). Free cells of the strain were routinely cultured and counted on MRS Agar at 37 °C for 48 h, under aerobic conditions.

### 2.3. Animals and treatments

All experimental procedures involving animals were approved by the "Comitato Etico-Scientifico per la Sperimentazione Animale" of the

University of Naples Federico II and were authorized by the Italian Health Ministry (137/2022-PR). This work complies with the animal ethic principles and regulations of the Italian Health Ministry. The authors ensured that all the experimental steps were taken to minimize the pain and suffering of the animals.

Male Wistar rats (Charles River, Calco, Lecco, Italy) of 90 days were caged in a temperature-controlled room ( $23 \pm 1$  °C) with a 12 h light/dark cycle (06.30 – 18.30 h).

The rats were divided in three groups and treated for 8 weeks with a control diet (C group, N = 8), or with a high fat - high fructose diet (D, N = 8 and DR groups, N = 8). In addition, DR rats daily received 0.5 mL of a 10 % sucrose solution containing  $10^8$  CFU of *L. reuteri* for the entire treatment period (8 weeks) while C and D rats received the same amount of sucrose solution without probiotics. Sucrose solution with or without probiotics was presented by an operator every day at the same hour through a needleless syringe and voluntarily consumed by rats. The composition of the two diets is shown in Supplementary Table S1. Body weight was monitored during the experimental period.

At the end of the experimental period, the rats were anesthetized with sodium pentothal ( $40 \text{ mg kg}^{-1}$  i.p.), euthanized by decapitation, and frontal cortex and ileum were harvested and dissected as previously described (Spagnuolo et al., 2020; Mazzoli et al., 2021). Freshly processed aliquots of frontal cortex from the different animal groups were used for the measurement of mitochondrial oxygen consumption, while pieces of each sample were immediately snap frozen in liquid nitrogen and stored at  $-80$  °C for further analyses or fixed for immunofluorescence.

### 2.4. Behavioral test

Behavioral analyses were carried out on rats 3 days before euthanasia. Behavioral experiments were conducted between 9:00 A.M. and 13:00 P.M. during the light phase of the 12/12 h light/dark cycle in dedicated testing sound-attenuated rooms. The apparatus used for the novel object recognition test consists of a sound-proof square arena ( $40 \text{ cm} \times 40 \text{ cm} \times 10 \text{ cm}$ ;  $l \times w \times h$ ). The rat behavior was recorded with a video camera. The procedure included three phases: habituation, training, and testing. Animals were brought to the testing room 30 min before the experiment to familiarize with the environment. During the habituation phase, the animal was individually placed in the middle of the empty arena for 5 min; subsequently, each rat took a training trial followed by a testing trial. During the training trial, each rat was individually placed into an open-field arena, containing two identical objects equidistant from each other, and allowed to explore the objects for 5 min. Thirty minutes later, the test session took place, during which one copy of the familiar object and a new object were placed in the same location as during the training trial. Each rat was placed in the apparatus for 5 min, and the time spent exploring each object was recorded. The objects used in this study were different in shapes and colors but identical in size. The objects were fixed on the floor of the box to avoid their movement. At the end of each test, the arena was sanitized to eliminate any odor that could interfere with the response of the following rat.

The location preference in the training phase and recognition index (RI) in the testing phase were calculated using the following formulas:

**Location preference** = Time exploring one of the identical objects/Time exploring the identical object pairs  $\times 100$  %.

**Recognition index (RI)** = Time exploring novel object/(Time exploring novel object + Time exploring familiar object)  $\times 100$  %.

Location preference was used as an environmental control, which should be 50 % to rule out the influence of the location of the object.

Rearing behavior was evaluated as stereotyped behavior and was defined as the lifting of the two front paws off the ground and with the body fully extended (Kleven et al., 1996). Observations were made during a 5 min period, and the behavior was considered present if the animal showed uninterrupted signs for at least 3 sec. Behavior was videotaped for the entire 5 min of each test and then analyzed off line by

the same researcher that carried the evaluation and that was blind to the condition to which the rat was assigned. Data are presented as duration (s) of time standing on hind legs with body fully extended.

All the above recordings were then later scored by independent raters that were blind to the experimental conditions.

## 2.5. Preparation of frontal cortex extracts

Aliquots of frozen frontal cortex (50 mg) were homogenized in seven volumes of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5 % sodium deoxycholate, 0.5 % NP-40, 0.1 % SDS pH 8.0) as previously reported (Spagnuolo et al., 2014). Nuclear extracts used for detecting Nrf2 translocation to the nucleus were isolated as previously reported (Zvonic et al., 2004).

## 2.6. Oxidative stress parameters

Nitro-tyrosine (N-Tyr) concentration was measured by ELISA in both plasma and frontal cortex samples diluted 1:8,000, 1:16,000, and 1:32,000 with coating buffer (7 mM Na<sub>2</sub>CO<sub>3</sub>, 17 mM Na-HCO<sub>3</sub>, 1.5 mM NaN<sub>3</sub>, pH 9.6), according to a previously published procedure (Spagnuolo et al., 2015). In details, aliquots (50 µL) of diluted samples were incubated (overnight, 4 °C) in the wells of a microtitre plate (Nunc MaxiSorp, distributed by VWR International, Milan, Italy). After four washes with T-TBS (130 mM NaCl, 20 mM Tris-HCl, 0.05 % Tween, pH 7.4) and four washes with high-salt TBS (500 mM NaCl, 20 mM Tris-HCl, pH 7.4), the wells were blocked with TBS containing 0.5 % BSA (1 h, 37 °C). After washing, the wells were incubated (1 h, 37 °C) with 50 µL of rabbit anti-N-Tyr antibody (CVL-PAB0188, Covalab, distributed by SIAL, Rome, Italy; 1: 1000 dilution in T-TBS containing 0.25 % BSA) followed by 60 µL of Goat anti-rabbit horseradish peroxidase-conjugated IgG (Immunoreagents, Raleigh, NC, USA; 1:5000 dilution in T-TBS containing 0.25 % BSA). Peroxidase-catalysed color development from o-phenylenediamine was measured at 492 nm. Results are reported as OD per mg of total proteins.

Lipid peroxidation was determined according to Fernandes et al. (2006) by measuring thiobarbituric acid-reactive substances (TBARS) (Fernandes et al., 2006). Aliquots of frontal cortex homogenates were added to 0.5 mL of ice-cold 40 % TCA. Then, 2 mL of a aqueous solution containing 0.67 % thiobarbituric acid and 0.01 % of 2,6-di-*tert*-butyl-p-cresol were added. The mixtures were heated at 90 °C for 15 min, then cooled in ice for 10 min and centrifuged at 850 x g for 10 min. The supernatant fractions were sampled and lipid peroxidation was spectrophotometrically measured at 530 nm. The amount of thiobarbituric acid-reactive substances formed was calculated using a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  and expressed as nmol thiobarbituric acid-reactive substances/g tissue.

SOD activity was measured in a medium containing 50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.8, 20 mM cytochrome c, 0.1 mM xanthine, and 0.01 units of xanthine oxidase. Determinations were carried out spectrophotometrically (550 nm) at 25 °C, by monitoring the decrease in the reduction rate of cytochrome c by superoxide radicals as generated by the xanthine-xanthine oxidase system. One unit of SOD is defined as the concentration of the enzyme that inhibits cytochrome c reduction by 50 % in the presence of xanthine + xanthine oxidase (Flohè & Otting, 1974).

Catalase activity was measured in 50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0, containing 10 mM H<sub>2</sub>O<sub>2</sub> and 0.25 % Triton X-100. Determinations were carried out spectrophotometrically (240 nm) at 25 °C, by monitoring the decrease in the absorbance due to the decomposition of H<sub>2</sub>O<sub>2</sub>. The rate of H<sub>2</sub>O<sub>2</sub> loss from the solution was linear when the natural log of the absorbance was plotted against time, due to the usual first-order kinetics exhibited by catalase (Maehly & Chance, 1954). Linear regression analysis was carried out to calculate the first-order reaction rate constant, and the resulting values were then referred to the unit of tissue weight.

Glutathione reductase (GR) activity was measured by monitoring the

decrease of NADPH absorbance at 340 nm, at 30 °C (Mazzoli et al., 2023). The reaction mixture contained potassium phosphate buffer, 2 mM EDTA, 2 mM NADPH (in 10 mM Tris-HCl, pH 7), and 20 mM oxidized glutathione. The activity was calculated using the NADPH molar extinction coefficient,  $\epsilon = 6.22 \times 10^{-5}$ , considering that one unit of GR is defined the amount of enzyme that catalyzes the reduction of 1 µmol of NADPH per minute. The specific activity was expressed in µU per g of tissue.

NADPH oxidase activity was assayed according to a modification of a method previously described (Bettaieb et al., 2014). Briefly, tissues (1:10 w/v) were homogenized in ice-cold Krebs buffer and then centrifuged at 800 x g, at 4 °C, for 10 min. The supernatant was collected and then centrifuged at 30,000 x g for 2 h at 4 °C. The pellet (membrane fraction) was re-suspended in Krebs buffer and the protein concentration was measured. Aliquots containing 100 µg of protein were added to Krebs buffer containing 500 µM NADPH. The change in absorbance at 340 nm was followed for 10 min, at 30 s intervals.

## 2.7. Mitochondrial analyses

Freshly isolated frontal cortex samples were homogenized (1:1000, w/v) in Mir05 medium containing 110 mM sucrose, 60 mM potassium-lactobionate, 20 mM Hepes, 20 mM taurine, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 6 mM MgCl<sub>2</sub>, 0.5 mM EGTA, and 0.1 % w/v fatty acid-free BSA, pH 7.0.

Homogenates (2 mg) were transferred into calibrated Oxygraph-2 k (O2k, Oroboros Instruments, Innsbruck, Austria) 2-mL chambers. Oxygen polarography was performed at  $37 \pm 0.001$  °C (electronic Peltier regulation), and oxygen concentration (µM) and oxygen flux (pmol O<sub>2</sub> s<sup>-1</sup> mL<sup>-1</sup>) were real-time recorded and corrected automatically for instrumental background by DatLab software (Oroboros Instruments, Innsbruck, Austria).

After addition of the homogenates, the O<sub>2</sub> flux was allowed to stabilize. A substrate, uncoupler, inhibitor titration (SUIT) protocol was applied to assess qualitative and quantitative mitochondrial changes (Burtscher et al., 2015). After stabilization, leak respiration supported primarily by electron flow through complex I of the respiratory chain was evaluated by adding the substrates malate (0.5 mM), pyruvate (5 mM), and glutamate (10 mM). Electron transfer was coupled to phosphorylation by the addition of 2.5 mM ADP, assessing phosphorylating respiration with electron transfer supported by complex I. Succinate (10 mM) was added to the chamber to induce maximal phosphorylating respiration with parallel electron input from complexes I and II. Oligomycin (2.5 µM) was added to assess leak respiration when substrates and ADP were provided, but ATP synthase is inhibited. Maximum capacity of the electron transport chain was obtained by addition of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 0.5 µM). Rotenone (0.5 µM) was added to inhibit complex I; hence, the maximal capacity supported by complex II alone was determined. Residual oxygen consumption was established by the addition of the inhibitor antimycin A (2.5 µM) and the resulting value was subtracted from the fluxes in each run, to correct for non-mitochondrial respiration. All samples were run in duplicates and the mean values were used for the analysis.

Procedures to test mitochondrial integrity were routinely carried out at the beginning of each measurement, by evaluating the stimulating effect of 10 mM exogenous cytochrome c on mitochondrial respiration in the presence of complex I-linked substrates and ADP.

## 2.8. Western blotting

Aliquots of frontal cortex proteins (30 µg) were fractionated by denaturing and reducing electrophoresis (Spagnuolo et al., 2018) on 12.5 % (to titrate Nrf2, synaptophysin, BDNF; cAMP response element-binding protein, CREB; superoxide dismutase, SOD; catalase; glutathione reductase, GR; respiratory mitochondrial complexes I-V, OXPHOS; Glucose transporter 5, Glut-5) or 10 % (Glycogen synthase kinase 3 beta, GSK; N-methyl-D-aspartic acid receptor, NMDAR; post-

synaptic density protein 95, PSD-95; synaptotagmin I, peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC-1 $\alpha$ ; uncoupling protein-2, UCP2; protein kinase B, Akt) polyacrylamide gels. Proteins blotting onto PVDF or nitrocellulose membrane (GE Healthcare; Milan, Italy), washing and blocking steps were carried out according to previously published procedures (Cigliano et al., 2018; Cigliano et al., 2019).

After blocking the membranes were incubated with primary antibodies (overnight, at 4 °C), washed and then treated (1 h, at 37 °C) with the appropriate peroxidase-conjugated secondary antibodies. The specific dilution of each antibody is shown in Supplementary Table S2. As the amount of phosphorylated Akt, GSK and CREB were expressed relative to total Akt, GSK and CREB level, respectively, after revelation of the immunocomplexes, the membrane was stripped (Spagnuolo et al., 2018) and then incubated with rabbit anti-Akt or mouse anti-GSK or rabbit anti-CREB (Supplementary Table S2). As the accurate quantification of each mitochondrial complex required the use of different dilutions of secondary antibody for optimizing band intensities (D'Ambrosio et al., 2023), we used GAM-HRP IgG diluted 1:90,000 for the detection of complex I, 1:70,000 for complex II, 1:150,000 for complex III, IV, and V. For loading control, after detection of each antigen, the membranes were stripped and incubated (overnight, 4 °C) with mouse anti- $\beta$ -actin IgG (1:1,000 in 0.25 % v/v non-fat milk) followed by GAM-HRP IgG (1:30,000 in 0.25 % v/v non-fat milk; 1 h, 37 °C). Nuclear amount of Nrf2 was assessed by using histone H3 for normalization (Supplementary Table S2). Signal detection was carried out using the Excellent Chemiluminescent Kit Westar Antares (Cyanagen s.r.l., Bologna, Italy). Densitometric analysis of chemidoc or digital images of X-ray films exposed to immunostained membranes was performed with Un-Scan-It gel software (Silk Scientific, UT, USA).

## 2.9. Evaluation of monoamine oxidase activity

Monoamine oxidase (MAO) activity was measured spectrophotometrically following the conversion of benzylamine to benzaldehyde, as previously described (Spagnuolo et al., 2023).

## 2.10. Immunofluorescence analysis

Paraffin embedded sections of frontal cortex from all the animal groups were stained with the phospho(Ser133)-cAMP response element-binding protein (p-CREB) specific monoclonal antibody (Ser 133) (87G-3) (Cell Signaling Technology; 1:1,000 in dilution in PBS containing 2 % w/v BSA; overnight, at 4 °C), and DAPI (Sigma Aldrich, Saint Louis, MO, USA). For the analysis, images were acquired with 40x magnification and 3 random fields/section per rat were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Images were captured and visualized using a Zeiss Confocal Microscope LSM 700 at 40x magnification, using a drop of immersion oil (Immersoil 518F, Zeiss).

## 2.11. Metabolic parameters

Glucose levels were measured with a commercial kit (glucose assay kit: catalogue n. 4058, GS Diagnostic) on frontal cortex and plasma samples taken after 6 h of fasting from a small tail clip. The amounts of fructose and uric acid in gut, plasma and frontal cortex samples were measured by colorimetric enzymatic methods, using commercial kits according to the manufacturer's instruction (Fructose assay kit: catalogue n. FA-20, Sigma Aldrich, St. Louis, MO, USA; Uric acid kit: catalogue n. 4059, GS Diagnostics SRL, Guidonia Montecelio, Rome, Italy).

## 2.12. Quantification of ketone bodies

Ketone bodies in rat plasma samples were quantified according to a previous procedure (García-Rivera et al., 2022), with minor modifications focused on the optimal chromatographic separation of hydrazone

derivatives through a core-shell C18 column. Briefly, 10  $\mu$ L of plasma was spiked with 1  $\mu$ L of SCFA carbon labelled internal standard mix including  $^{13}\text{C}_2$ -acetate,  $^{13}\text{C}_3$ -propionate and  $^{13}\text{C}_4$ -butyrate (final concentration 0.05 mM for each compound). Plasma proteins were precipitated with the addition of 60  $\mu$ L of 75 % v/v methanol, while derivatization was achieved through the mixing of suspensions with 60  $\mu$ L of 3-NPH (200 mM) and 10  $\mu$ L of EDC (120 mM in 6 % pyridine). Upon incubation at room temperature (22 °C) for 45 min under gentle shaking in an orbital shaker, derivatization reaction was stopped with the addition of 10  $\mu$ L quinic acid (200 mM). Samples were centrifuged at 15,000 rpm for 5 min at 4 °C, and supernatants diluted up to 1 mL with 10 % v/v methanol. Samples were centrifuged again at 4 °C, for 5 min, at 15,000 rpm, then analyzed without any further dilution by liquid chromatography-high resolution mass spectrometry. Quantitation of ketone bodies and SCFA hydrazone derivatives was achieved by a U-HPLC system (Ultimate 3000 RS, Thermo Fisher Scientific) interfaced to a linear ion trap hybrid Orbitrap high resolution mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific). Chromatographic separation encompassed the use of a Kinetex C18 PS column (100 x 2.1 mm, 2.6  $\mu$ m; Phenomenex, Torrance, CA), thermostated at 40 °C; analytes were eluted with the following gradient of solvent B (minutes/%B): (0/5), (5/5), (12.3/35), (13.3/85), (14/99), (16/99). Mobile phases consisted of water (solvent A) and acetonitrile (solvent B), and the flow rate was 0.2 mL/min. LC stream was interfaced to an electrospray ion source (ESI) working in negative ion mode, scanning the ion in the  $m/z$  range 100–400; resolution was set at 30,000 (FWHM at  $m/z$  200), capillary temperature was 300 °C, while sheath and auxiliary gases were set at 25 and 15 arbitrary units, respectively. Analyte profile data in full MS mode were collected using Xcalibur 2.1 (Thermo Fisher Scientific). Calibration curve was obtained with the internal standard technique in the linearity range 0.001–1 mM by using the same procedure detailed above for plasma samples. Wherever the respective internal standard was not available, chemical similarity was used to select the appropriate compounds for recovery assessment and matrix effect minimization:  $^{13}\text{C}_4$ -butyric acid was used as internal standard for  $\beta$ -hydroxybutyrate, and  $^{13}\text{C}_2$ -acetic acid and  $^{13}\text{C}_3$ -propionic acid were used as internal standards for acetoacetic acid. Analytical performances are detailed in Supplementary Table S3. Along with analytical reference standards matching, hydrazone derivative identity was further confirmed by product ion scan experiments set up on an Exploris 120 hybrid quadrupole high resolution mass spectrometer by using the same chromatographic profile described for quantitation experiments. Specifically, the analyzer resolution was set at 60,000 (FWHM at  $m/z$  200), working in the scan  $m/z$  range 100–400. Product ion scan properties included Orbitrap resolution at 60,000 (FWHM at  $m/z$  200) and the quadrupole resolution was set at 1. H-ESI parameters were as follows: negative ion static spray voltage was  $-3.2$  kV, ion transfer tube and vaporizer temperature were set at 320 °C and 300 °C, respectively; sheath gas flow and auxiliary gas flow were 45 and 10 arbitrary units, respectively.

## 2.13. Statistical analysis

Data were expressed as mean values  $\pm$  SEM. The program GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA) was used to verify normal distribution of data and to compare groups with one-way ANOVA followed by Bonferroni post-test.  $P < 0.05$  was considered significant in the reported analyses.

## 3. Results

### 3.1. Body weight and plasma glucose

Body weight time course is reported in Supplementary Fig. S1, showing that Western diet elicited a significant increase in body weight gain both in D and DR rats. No variation in fasting plasma glucose levels was evidenced in D and DR rats compared to C rats ( $C = 120 \pm 5$  mg/dL;

D = 124±5 mg/dL; DR = 118±4 mg/dL).

### 3.2. Behavioral analysis

We performed the novel object recognition (NOR) task to reveal diet-linked deficits in episodic and recognition memory and found that the novel object recognition index was significantly decreased in the WD-fed rat group (D) compared with the control ones (C), while the concomitant administration of WD-fed animals with *L. Reuteri* (DR) prevented this alteration (Fig. 1B). Location preference was used as an environmental control and no difference was found between the 3 groups of rats (Fig. 1A). The total exploration time of objects during the testing phase was comparable among the different animal groups (data not shown).

We also examined another unconditioned behavioral effect, i.e., rearing. WD feeding induced a significant increase in the rearing time in D rats compared to that of C counterparts; this alteration was counteracted by the concomitant administration of *L. reuteri* (DR rats) (Fig. 1C).

Notably, the administration to control rats (C) of *L. reuteri* (CR group; N = 8) had no effect on behavioral tasks, as no differences in the recognition index or rearing time were observed between C and CR rats (Supplementary Fig. S2).

### 3.3. Neurotrophins and synaptic proteins

The working memory is modulated by different molecules in cortex. Among these key players, we evaluated the protein level of the glutamate receptor NMDAR, which was found reduced following WD in the frontal cortex of D rats, and this decrease was prevented by concomitant *L. reuteri* administration (Fig. 2A). Further, the extent of phosphorylation of CREB, which regulates transcription and translation of proteins/receptors required for memory and synaptic plasticity (Impey et al., 2004; Barco et al., 2006), including BDNF, was reduced in D rats compared to C. Also in this case, the treatment with the probiotic was effective in preventing this alteration (Fig. 2B, Supplementary Fig. S3). In agreement with the decrease of the activating phosphorylation of CREB, a significant WD-dependent decrease of BDNF, a key cerebral factor involved in a wide range of neurophysiological processes (Kowiański et al., 2018), was observed in D rats (Fig. 2C). Notably, the alteration of BDNF was prevented by the concomitant administration of *L. reuteri*. Given the role played by BDNF in synaptic transmission, we further evaluated the amounts of two pre-synaptic proteins, namely synaptophysin and synaptotagmin I (Fig. 2D, E), and the post-synaptic protein PSD-95 (Fig. 2F), which play a relevant role in synaptic plasticity (Won et al., 2017). WD led to decreased levels of all three proteins, while the concomitant treatment with the probiotic prevented the alteration in these synaptic markers (Fig. 2D-F).

### 3.4. Oxidative balance

To investigate whether WD affects the redox homeostasis in frontal

cortex, lipid peroxidation as marker of oxidative damage to lipids, and N-Tyr as marker of oxidative damage to proteins, were evaluated in this brain compartment. Enhanced oxidative damage to proteins and lipids was observed in WD-fed rats. Interestingly, the concomitant treatment with *L. reuteri* in DR rats was associated to a significant decrease of both oxidation markers (Fig. 3A, B). Similar results in both TBARS and N-Tyr were revealed in plasma samples of C, D and DR rats (Supplementary Fig. S4).

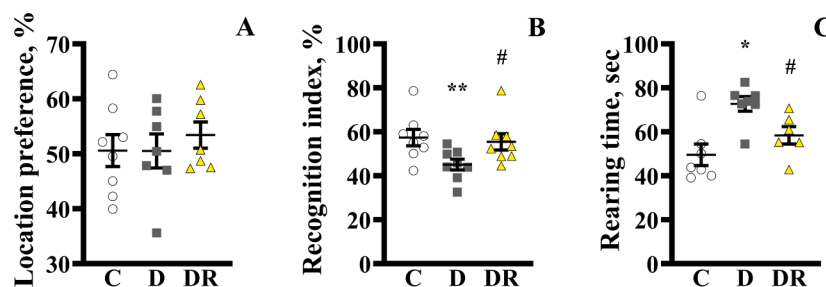
In line with the condition of oxidative stress in the frontal cortex of WD-fed rats, we observed a significant increase of critical oxidant-producing enzymes, namely NADPH oxidase and MAO (Snezhkina et al., 2019), while the activity of these enzymes was unaltered in DR rats, namely WD-fed rats that received concomitant administration of *L. reuteri* (Fig. 3C, D). Also, a WD-related decrease of two proteins, both playing a role in contrasting oxidative stress (Kumar et al., 2022; Shalbayeh et al., 2023), namely UCP2 and PGC-1 $\alpha$ , was observed in frontal cortex of D rats compared to C rats, while the above decrease was prevented, in DR rats (Fig. 3E, F), by the concomitant administration of *L. reuteri*.

### 3.5. Mitochondrial activity and cortex metabolism

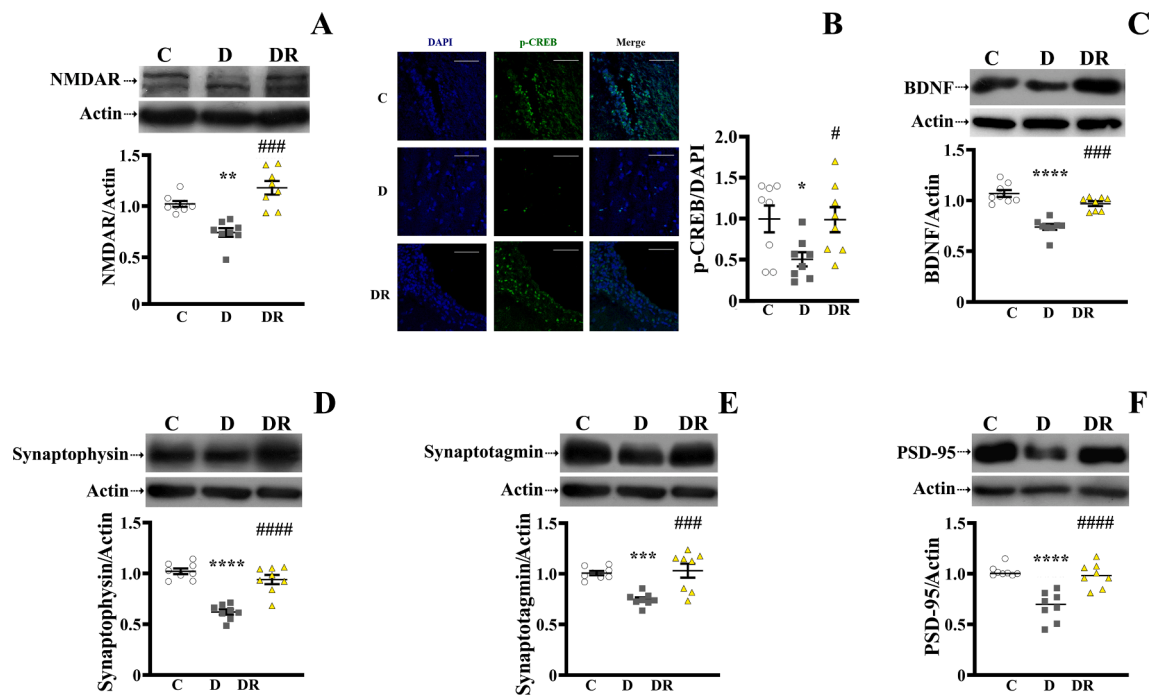
Since redox balance is strictly linked to mitochondrial function, the mitochondrial oxidative capacity was also analyzed. Unexpectedly, cortex mitochondria displayed a diet-induced increase in oxidative capacity which was not modified by the probiotic (Fig. 4A), although no significant variation in the amount of respiratory complex I, II, III, IV or V was measured (Fig. 4B). The increased mitochondrial oxidative capacity could be an adaptive mechanism to maintain cerebral energetics in face of changes in fuel availability. Although glucose concentrations in plasma were not different between the 3 groups of rats, cortex levels of glucose were found increased in WD-fed rats, thus suggesting a condition of brain glucose hypometabolism (Fig. 5A). The administration of WD-fed rats with *L. reuteri* maintained the frontal cortex glucose at levels comparable to those of the controls (Fig. 5A). Interestingly, plasma ketone bodies acetoacetate and  $\beta$ -hydroxybutyrate were significantly higher in D rats compared to C rats (Fig. 5B, C). The WD-induced increase of acetoacetate level was prevented by the treatment with *L. reuteri* (Fig. 5B). This metabolic fuel profile was coupled to a significantly lower degree of inhibitory phosphorylation of GSK in D rats compared to controls, which was prevented in DR rats (Fig. 5D). In contrast to the regulation of GSK, we observed a significant activation of the Akt pathway, expressed as p-Akt/Akt ratio, in D group (Fig. 5E). This phenomenon was not observed in DR rats that experienced WD plus *L. reuteri*.

### 3.6. Analysis of the Nrf2 pathway

The pathway of Nrf2, the key transcriptional activator of genes responsible for the maintenance of redox homeostasis (Heurtaux et al.,



**Fig. 1.** Location preference (A), Recognition index (B) and rearing time (C) during Novel Object Recognition test in control (C), Diet (D) and Diet-Reuteri (DR) rats. Location preference was calculated as the percentage of time spent in exploring one of the two identical objects within the training session. The Recognition index was calculated as the percentage of time spent exploring the novel object in the testing session. Values are the means  $\pm$  SEM of 8 different rats. \* $P < 0.05$ , \*\*  $P < 0.01$  compared to C rats; # $P < 0.05$  compared to D rats (one-way ANOVA followed by Bonferroni post-test).



**Fig. 2.** Western blot quantification (with representative blots) of N-methyl-D-aspartic acid receptor (NMDAR) (A), Brain derived neurotrophic factor (BDNF) (C), synaptophysin (D), Synaptotagmin (E), PSD-95 (F), and immunofluorescence quantification (magnification 40x, scale bar=100 $\mu$ m) of phospho-cAMP response element-binding protein (p-CREB) (B) in frontal cortex from control (C), Diet (D) and Diet-Reuteri (DR) rats. Values are the means  $\pm$  SEM of 8 different rats. \* $P$ <0.05, \*\*  $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\*  $P$ <0.0001 compared to C rats; # $P$ <0.05, ### $P$ <0.001, #### $P$ <0.0001 compared to D rats (one-way ANOVA followed by Bonferroni post-test).

2022), was investigated in brain cortex of different rat groups. The nuclear protein level of Nrf2 was found reduced by the WD, while the concomitant administration of *L. reuteri* prevented this decrement (Fig. 6A).

Both protein levels and activities of Nrf2 pathway-modulated enzymes catalase, GR and SOD, were measured. SOD and GR levels were reduced in D rats and the treatment with *L. reuteri* was able to prevent these alterations in DR rats (Fig. 6B, C). No changes of catalase protein level were associated with WD treatment (Fig. 6D).

Also, a parallel decrease of the activity of SOD, catalase and GR was associated with WD (Fig. 7A-C). While administration of *L. reuteri* in WD-fed rats (DR) was found effective in preventing diet-induced changes of both SOD and GR activities, no similar effect was observed for the catalase activity.

### 3.7. Fructose and uric acid level in Gut, plasma and cortex

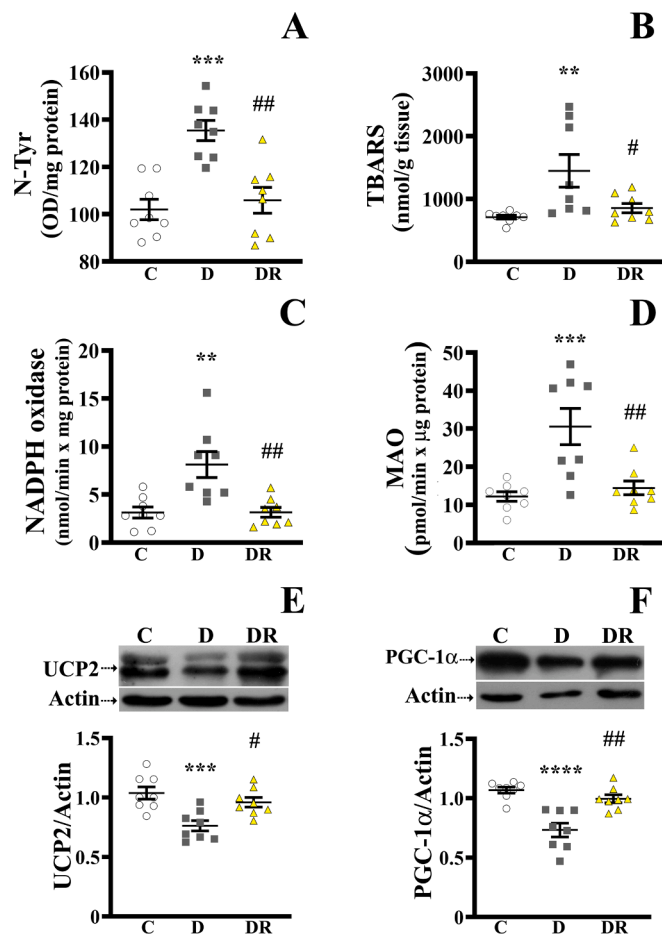
To delineate the mechanism underlying brain alteration of redox homeostasis we focused on the role of fructose. To obtain information on the absorption of fructose into the gut and its metabolism, as well as its circulating levels and delivery to the brain cells, we quantified the levels of fructose and uric acid, one of the main products of fructose metabolism, in the ileum of C, D and DR rats. Significant increases in ileum levels of fructose and uric acid were found in D rats compared to C rats, while this increase was prevented in DR rats (Figure 8A, B). In line with this result, the amount of fructose in plasma of D rats was higher compared to C, but no difference was measured between C and DR rats (Fig.8C). Moreover, the levels of the sugar were also higher in the frontal cortex of D rats respect to C animals, with the probiotic *L. reuteri* being effective in preventing fructose changes (Fig.8E). Accordingly, the amount of fructose specific transporter Glut-5 was found increased in D rats compared to C rats, but no difference was detected between C and DR rats (Supplementary Fig. 5).

## 4. Discussion

The present study deals with the critical issue of the ability of the probiotic *L. reuteri* to modulate western diet-induced alterations in brain function. Although different public awareness campaigns are underway to discourage the adoption of unhealthy diets rich in fats and/or sugars, less attention is paid to the search for strategies to limit the corresponding brain damage. In this frame, the relationships between diet-related changes in the gut-brain axis and cognitive flexibility has now emerged as a turning point for promoting healthy dietary regimen (Dinan and Cryan, 2017; Schächtle and Rosshart, 2021; Boehme et al., 2023). Nevertheless, while the impact of prebiotics or plant-derived metabolites on brain cognition has been deeply investigated in recent years (Kennedy, 2014; Chu et al., 2023; Varesi et al., 2023), few data are available on the efficacy of probiotics in contrasting diet-induced brain dysfunction (Ohland et al., 2013; Jena et al., 2022). In this study, we provide the first experimental evidence that *L. reuteri* supplementation ameliorates WD-related cognitive impairment, as assessed behaviorally and at molecular level in the frontal cortex. This result is of interest taking into account that we recently reported that the *L. reuteri* administration in western diet-fed rats does not modify the microbiota (Abuqwider et al., 2023).

The NOR test, one of the most employed approaches to examine working memory (Ennaceur et al., 1997; Ennaceur and Delacour, 1988) was used to evaluate short-term memory. The recognition of the novel object was significantly decreased in D rats, suggesting an impairment of the object memory induced by WD. This alteration was not due to changes in exploratory motivation during the sampling phase of the task, since exploration of both objects is identical for both groups (Akerman et al., 2012). Interestingly, *L. reuteri* supplementation was effective for the improvement of learning-related memory cognition.

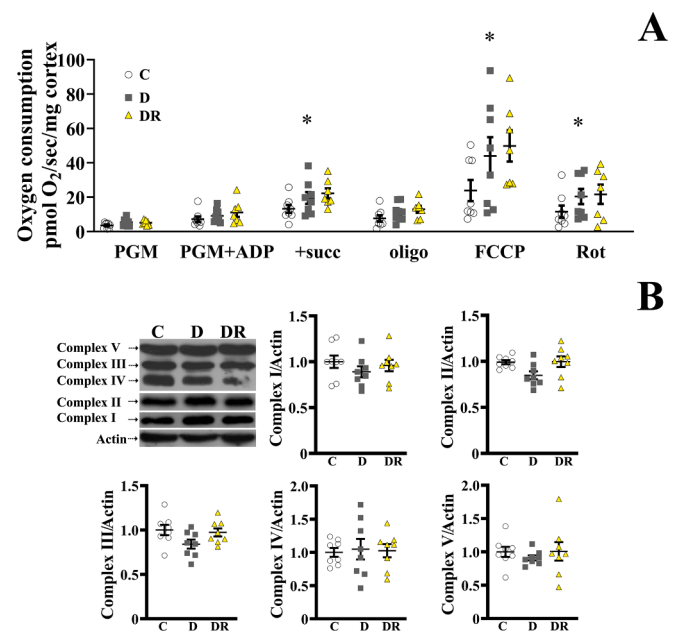
The working memory, which relates to the 'temporary operation and storage of information', is mainly stored in the prefrontal cortex of the brain (Khan and Muly, 2011), where NMDA receptor, once activated,



**Fig. 3.** Quantification of Nitro-tyrosine (N-Tyr) (A), and thiobarbituric acid reactive substances (TBARS) (B), NADPH oxidase (C) and Monoamine oxidase (MAO) (D) activities, and Western blot quantification (with representative blots) of Uncoupling protein 2 (UCP2) (E) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) (F) in frontal cortex from control (C), Diet (D) and Diet-Reuteri (DR) rats. Values are the means  $\pm$  SEM of 8 different rats. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  compared to C rats; #  $P < 0.05$ , ##  $P < 0.01$ , compared to D rats (one-way ANOVA followed by Bonferroni post-test).

leads to the conversion of the transcription factor CREB into its active form, namely p-CREB (Miyamoto, 2006; Yan et al., 2016), which initiates transcription and translation of proteins/receptors required for memory and synaptic plasticity (Impey et al., 2004; Barco et al., 2006), including BDNF that is critical for neuronal circuitry function. Here, we report that *L. reuteri* supplementation prevents the WD-induced decrease of frontal cortex levels of NMDA receptor, p-CREB and BDNF. Hence, we can speculate that the protective effect of the probiotic on the levels of these players contributed to preserve neuronal plasticity, learning and memory of WD-fed rats.

Besides trophic factors, presynaptic proteins are important molecules regulating cognitive and other complex functions in the frontal cortex. In this context, worth mentioning are synaptophysin, which is involved in the regulation of the kinetics of synaptic vesicle endocytosis (Kwon and Chapman, 2011), synaptotagmin I, a major calcium sensor for transmitter release (Courtney et al., 2019) and PSD-95, pivotal for the protein scaffolding in excitatory neurons (Pinto et al., 2013). The levels of these synaptic markers were reduced in adults D rats, in agreement with results previously obtained in the brain of middle-aged rats fed a western diet (Mazzoli et al., 2020). The finding of decreased amounts of synaptic proteins, together with behavioral changes in D rats, is in good accordance with the previous results showing that PSD-95 deficiency

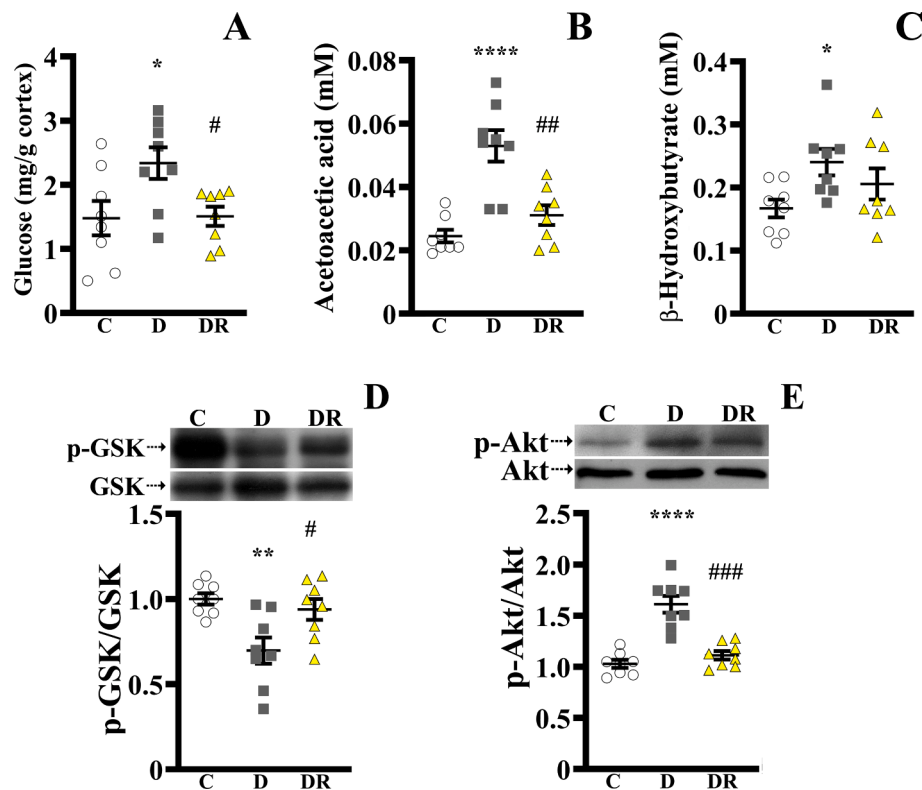


**Fig. 4.** Mitochondrial respiratory activity (A) and Western blot quantification (with representative blots) of mitochondrial complexes (B) in frontal cortex from control (C), Diet (D) and Diet-Reuteri (DR) rats. Mitochondrial respiration (panel A) was measured in the presence of complex I-linked substrates pyruvate + glutamate + malate (PGM), adenosintriphosphate (ATP), complex II-linked substrate succinate (S), ATP synthase inhibitor oligomycin (O), uncoupler carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP) and inhibitor of complex I rotenone (R). Values are the means  $\pm$  SEM of 8 different rats. \*  $P < 0.05$ , compared to C rats (one-way ANOVA followed by Bonferroni post-test).

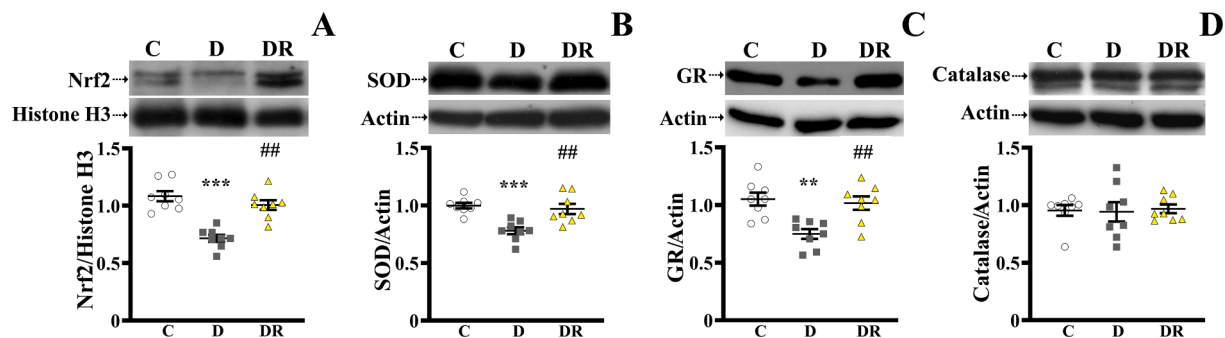
disrupts cortex-associated function and behavior, particularly learning and working memory (Coley and Gao, 2019). The dysfunction of BDNF and PSD-95 could also be at the basis of the increased rearing (index of stress condition) found in D rats, since these mediators are known to be involved in anxiety-like behavior (Feyder et al., 2010; Murinová et al., 2017). Interestingly, the *L. reuteri* supplementation was able to totally prevent changes in the levels of all three proteins in DR rats. Overall, it is conceivable that *L. reuteri*-induced increase in the activation of CREB, in the levels of BDNF and synaptic proteins underlies the maintenance of the cognitive status of WD plus probiotic-fed rats.

One of the main culprits for the cognitive dysfunction associated with an unbalanced diet is the alteration of brain redox homeostasis (Ionescu-Tucker and Cotman, 2021; Sharma, 2021). In agreement, in the current study, we observed that impaired behavioral performance in WD-fed rats is associated with increased protein and lipid oxidation. The higher degree of oxidative damage was found associated, in D rats, with the concomitant reduction of the levels of UCP2 and PGC-1 $\alpha$ , two key factors for neuroprotection, antioxidant response and synaptic plasticity (Valle et al., 2005; Cheng et al., 2012; Hermes et al., 2016). The observed decrease of PGC-1 $\alpha$  was also in line with the alteration of synaptic proteins and BDNF levels, as BDNF expression is regulated by PGC-1 $\alpha$  (Xia et al., 2017). All observed alterations were fully prevented in WD-fed rats concomitantly receiving the probiotic.

Although mitochondrial dysfunction is often found at the basis of oxidative imbalance, an unexpected increase in mitochondrial respiratory capacity was evidenced in the frontal cortex of D rats. One possible explanation could be that this increase represents an adaptive mechanism to maintain cerebral energetics in response to alterations in cortex metabolic activity, since it has been shown that BDNF levels correlate with glucose oxidation in cortical neurons (Burkhalter et al., 2003). Indeed, the levels of cortex glucose were found increased in WD-fed rats, thus suggesting a condition of glucose hypometabolism, in



**Fig. 5.** Glucose cortex content (A), plasma levels of acetoacetic acid (B) and  $\beta$ -hydroxybutyrate (C), Western blot quantification (with representative blots) of phospho-Glycogen synthase kinase 3 beta (pGSK) (D), and phospho-protein kinase B (pAkt) (E) in frontal cortex from control (C), Diet (D) and Diet-Reuteri (DR) rats. Values are the means  $\pm$  SEM of 8 different rats. \* $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*\*  $P < 0.0001$  compared to C rats; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  compared to D rats (one-way ANOVA followed by Bonferroni post-test).



**Fig. 6.** Western blot quantification (with representative blots) of nuclear factor -erythroid 2-related factor 2 (Nrf2) (A), superoxide dismutase (SOD) (B), glutathione reductase (GR) (C), and catalase (D) in frontal cortex from control (C), Diet (D) and Diet-Reuteri (DR) rats. Values are the means  $\pm$  SEM of 8 different rats. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  compared to C rats; ## $P < 0.01$ , compared to D rats (one-way ANOVA followed by Bonferroni post-test).

concomitance with a reduced phosphorylation of GSK, which is a downstream marker of insulin signaling. A condition of decreased glucose utilization is usually associated with an increased utilization of ketone bodies (Giménez-Cassina et al., 2012). Interestingly, both plasma ketone bodies acetoacetate and  $\beta$ -hydroxybutyrate were higher in D rats compared to C rats. Indeed, elevated plasma levels of the ketone bodies acetoacetate and  $\beta$ -hydroxybutyrate were already observed in untreated diabetic patients or high fat fed animals (Andersen et al., 2017; Sikder et al., 2018; Yamasaki et al., 2023), in line with results obtained in our experimental paradigm. Concomitant probiotic administration in WD-fed rats fully restored glucose utilization by cortical cells, probably through the maintenance of BDNF levels, and totally reestablished the plasma levels of acetoacetate, even though  $\beta$ -hydroxybutyrate concentrations were reduced with respect to WD-fed animals but remained higher compared to controls. These latter results open an interesting

perspective in which mitochondrial function and oxidative balance are not necessarily linked, at least in the brain likely because maintaining ATP production in this tissue is of priority compared to other tissues in which mitochondrial dysfunction and oxidative stress are very often found after nutritional insult (García-García et al., 2020).

ROS can be also produced in the cytosol by a large panel of active oxidoreductases including NADPH oxidase and MAO (Snezhkina et al., 2019; Heurtaux et al., 2022). Indeed, we found increased activity of both enzymes in D rats compared to controls, suggesting that, in our experimental paradigm, they represent relevant promoters of free radical production. Furthermore, Akt activation was observed in the frontal cortex of rats receiving the western diet. This result well agrees with previous evidence describing the role of this kinase in activating NADPH oxidase (Chen et al., 2003; Hoyal et al., 2003; Zhang et al., 2016). Consistent with the prevention of oxidative damage exerted by *L. reuteri*,



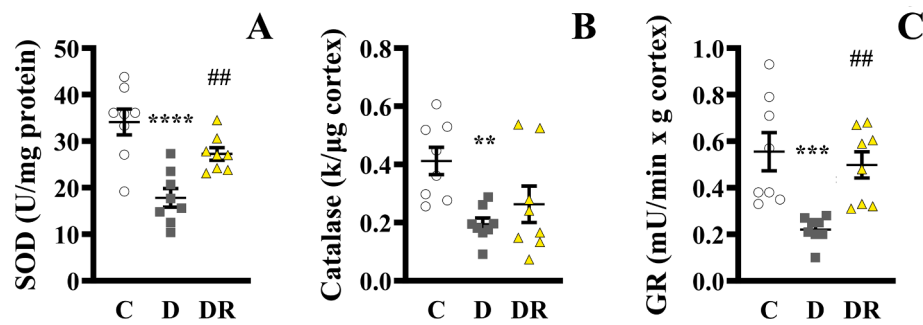


Fig. 7. Enzymatic activity of superoxide dismutase (SOD) (A), catalase (B) and glutathione reductase (GR) (C), in frontal cortex from control (C), Diet (D) and Diet-Reuteri (DR) rats. Values are the means  $\pm$  SEM of 8 different rats. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  compared to C rats; ##  $P < 0.01$ , compared to D rats (one-way ANOVA followed by Bonferroni post-test).

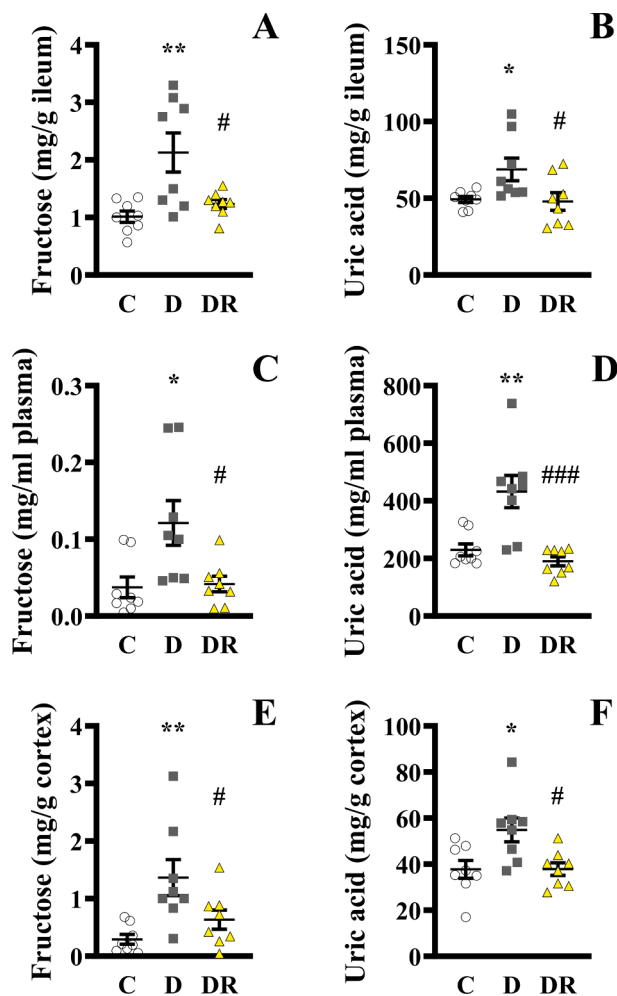


Fig. 8. Fructose and uric acid levels in ileum (A and B respectively), plasma (C and D respectively), and frontal cortex (E and F respectively) from control (C), Diet (D) and Diet-Reuteri (DR) rats. Values are the means  $\pm$  SEM of 8 different rats. \*  $P < 0.05$ , \*\*  $P < 0.01$ , compared to C rats; #  $P < 0.05$ , ###  $P < 0.001$  compared to D rats (one-way ANOVA followed by Bonferroni post-test).

all the above increases were not detected in DR rats.

Our results highlighted a role for decreased Nrf2 signaling as a local mediator of WD-induced oxidative stress within the brain. Nrf2 is a key regulator of the antioxidant response system, being activated in settings of oxidative damage and promoting increased antioxidant enzyme activity, thus being a promising target for the treatment of brain diseases (Hashimoto, 2018; Urano and Yamamoto, 2023). In fact, we found that

increased oxidative stress following the WD was associated with impaired Nrf2 signaling, with following decreased activities/amounts of SOD, CAT and GR in D rats. In addition, the present study provides, for the first time, direct evidence that *L. reuteri* exerts a protective role against brain dysfunction associated with the WD consumption, at least partly by increased translocation into the nucleus of the redox transcription factor Nrf2 accompanied by higher expression of the downstream antioxidant enzymes.

To get insight into the possible mechanism through which *L. reuteri* administration influenced brain physiology and considering that the probiotic had no effect on the microbiota reshaping (Abuqwyder et al., 2023), we focused on the well-known prooxidant effect of fructose and its metabolites (Spagnuolo et al., 2020), already shown by us in a model of fructose-induced cortex dysfunction (Spagnuolo et al., 2023). It was previously outlined that excessive dietary fructose intake increases reactive species and oxidative damage, downregulates Nrf2 and blocks its antioxidant pathway (Jaiswal et al., 2015; Garcia-Arroyo et al., 2019; Batandier et al., 2020). Our group also previously reported that a fructose-rich diet reduced the Nrf2 protein content and the activity of Nrf2-dependent enzymes, namely G6PD and GSR, in the brain cortex of young and adult rats (Spagnuolo et al., 2020). Indeed, due to the uncontrolled metabolism of fructose in the cells, ATP can be depleted resulting in an activation of adenosine monophosphate deaminase, and subsequently, the production of the prooxidant uric acid (Staltnier et al., 2023), which activates NADPH oxidase (Lanaspa et al., 2012; Sanchez-Lozada et al., 2012) and causes a burst of intracellular oxidative stress (Johnson et al., 2020). Further, high fructose decreases miRNA-200a, a non-coding RNA, that is reported to activate Nrf2, thus inhibiting the Nrf2 antioxidant response (Zhao et al., 2018).

We hypothesized that the probiotic acted essentially by metabolizing part of the fructose transiting the gastrointestinal tract and thus limiting its absorption and delivery to peripheral organs (including brain) considering that it has been recently reported that *L. reuteri* utilizes fructose as a source of reducing power (Oh et al., 2019). To test this, we evaluated the content of fructose and its metabolite uric acid in ileum, and we found that their concentrations significantly increased after WD. These changes were not observed in probiotic-treated rats, thus indicating that fructose entry in the ileum was limited. In line, also the plasma levels of fructose were increased in D rats compared to C rats but were lower in DR ones. Consequently, the amount of fructose reaching the brain and converted in uric acid in frontal cortex was found increased by WD but significantly reduced in probiotic-treated rats. Accordingly, the amount of the specific fructose transporter Glut-5 raised in D rats with respect to C, and this increase was prevented by probiotic treatment, in agreement with the reduction in fructose found in the brain. Therefore, from this set of data it emerges that the ability of the probiotic to metabolize fructose in the gastrointestinal tract can limit sugar absorption, thus preventing uric acid increase and the following oxidative stress of cortex cells (Figure 9). The intricate connection

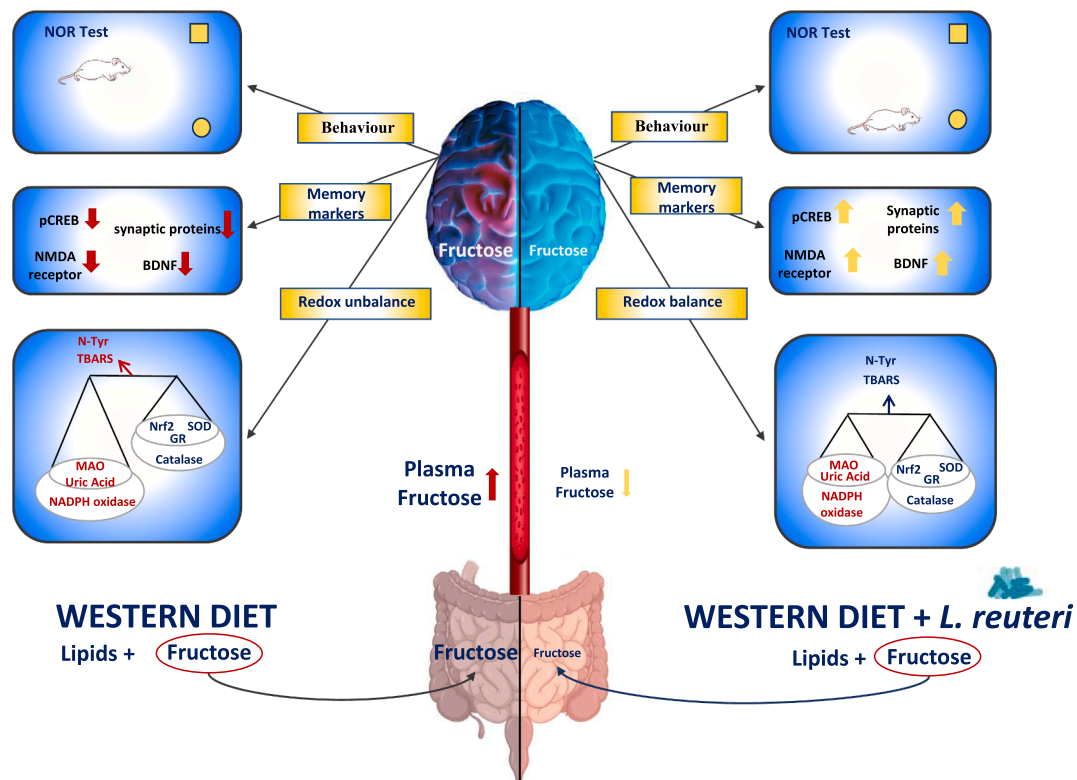


Fig. 9. *L. reuteri* impact on brain health of western diet-fed rats

between dietary habits, gut microbiota, fructose metabolism and brain dysfunction can be tackled through the fine tuning of the microbial population. These results open new scenarios for an integrated intervention study based on the molecular effects of microbial metabolites and their mechanistic effects on brain function.

In this context we believe that these results pave the way to dive deep into the probiotics preventive effect against brain dysfunction associated with unhealthy eating habits, which are typical of today's society.

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## CRediT authorship contribution statement

**Arianna Mazzoli:** Writing – review & editing, Supervision, Project administration, Investigation, Formal analysis, Conceptualization. **Maria Stefania Spagnuolo:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis. **Martina Nazzaro:** Investigation. **Angela Di Porzio:** Investigation. **Valentina Barrella:** Investigation. **Antonio Dario Troise:** Investigation, Formal analysis. **Sabrina De Pascale:** Investigation, Formal analysis. **Andrea Scaloni:** Supervision, Funding acquisition, Formal analysis. **Gianluigi Mauriello:** Resources, Investigation. **Susanna Iossa:** Writing – review

& editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Luisa Cigliano:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization.

## 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.

## Data availability

Data will be made available on request.

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## Data availability.

The data that support the findings of this study are available from the corresponding author upon request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2024.106162>.

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