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Protective effect of probiotic *Limosilactobacillus reuteri* DSM17938 against western diet-induced obesity and associated metabolic alterations

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ABSTRACT

Purpose: To evaluate the possible application of *Limosilactobacillus reuteri* DSM 17938 as a strategy to prevent Western Diet-induced metabolic alterations and liver dysfunction.
Methods: Male Wistar rats of 90 days were divided in three groups and fed a control diet (CD), a high fat - high fructose diet alone (WD) or in combination with the administration of 10^8 CFU of *L. reuteri* (WD-R) for 8 weeks. Body composition, energy balance and plasma lipid profile were evaluated, together with hepatic glucose homeostasis and insulin sensitivity. Metabolic inflammation was also assessed at a whole-body level and in the liver, together with mitochondrial function and oxidative stress.
Results: Rats that received the WD and *L. reuteri* administration exhibited a lower body lipid gain and a higher protein gain, underlying a beneficial effect of the probiotic in counteracting the development of obesity. Moreover, the WD-R rats were protected from the development of inflammation at a whole body and liver level and displayed normal glucose homeostasis and insulin sensitivity, a decreased hepatic lipid deposition and a preserved function of the mitochondrial respiratory chain and redox balance.
Conclusions: We demonstrate for the first time that *Limosilactobacillus reuteri* DSM 17938 has a strong efficacy in preventing the development of the hepatic metabolic derangement elicited by the Western diet administration. These are promising results that open the way for the use of this probiotic as a prevention strategy against the development of diet-related diseases, such as type two diabetes and metabolic syndrome.

1. Introduction

The prevalence of obesity is increasing worldwide, representing an alarming global public health issue. Several countries have witnessed an escalation in the prevalence of obesity in the last decades, probably due to urbanization, sedentary lifestyle, and increase consumption of high-calorie processed food (Hruby & Hu, 2015). The World Health Organization reports that 1.9 billion adults were overweight in 2016 and 650 million were considered obese (World Health Organization, 2021). One of the most prevalent obesity-associated metabolic diseases is type 2 diabetes (T2D), now a global pandemic and the number of patients that will be diagnosed with this disease is expected to further increase over the next decade (Unnikrishnan, Pradeepa, Joshi, & Mohan, 2017). Additionally, metabolic alterations, such as T2D and insulin resistance, frequently occur in conjunction with a spectrum of liver abnormalities starting from steatosis and progressing to lobular inflammation, cirrhosis and liver cancer (Friedman, Neuschwander-Tetri, Rinella, & Sanyal, 2018; Hardy, Oakley, Anstee, & Day, 2016). Non-alcoholic fatty liver disease (NAFLD) is the major cause of chronic liver disease globally, with a prevalence of 95% among those with diabetes and obesity and 20–40% in the general population (Levene & Goldin, 2012).

An unhealthy lifestyle and the corresponding change in gut microbiota result in the production of many pathogenic factors which significantly impact liver immunology and homeostasis, contributing to

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NAFLD pathogenesis (Leung, Rivera, Furness, & Angus, 2016), since the gut and the liver have close anatomical and functional communication through their connection via the portal vein. In particular, the so-called "western diet" (WD, a diet high in saturated fats and refined sugars) has been recognized as one of the major culprits of T2D (Ussar et al., 2015) and NAFLD, and gut microbiota is believed to play an important role in modulating the effects of this diet (Lazar et al.; Yang et al., 2023). At the moment, lifestyle modifications, including calorie restriction, exercises, and weight loss, are considered effective treatments for NAFLD (Kwak & Kim, 2018). However, these lifestyle changes have proven difficult to adhere to in routine clinical practice and therefore there is an urgent need to identify alternative approaches. Probiotics – defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Cremon, Barbaro, Ventura, & Barbara, 2018) - exhibit promising preclinical potential to reduce obesity, NAFLD, and insulin resistance. Probiotics are therefore considered a noninvasive yet potentially highly effective therapeutic approach against obesity-associated metabolic diseases (Delzenne, Neyrinck, Bäckhed, & Cani, 2011; Wiciński, Gębalski, Gołębiewski, & Malinowski, 2020). Notwithstanding this, evidence from clinical trials is still inconsistent (Editorial, 2019; Hill et al., 2014) and this emphasizes a critical need for an enhanced molecular understanding of strain- specific capabilities (Krautkramer, Fan, & Bäckhed, 2019).

Interestingly, a recent paper demonstrated that two Lactobacilli exerted a functional role in regulating metabolism by acting on mitochondrial function in the liver and leading to the improvement in hepatic lipid and systemic glucose metabolism (Rodrigues et al., 2021). *Limosilactobacillus reuteri* DSM 17938 is a probiotic bacterium, that has proved to be safe and effective in preventing and treating numerous gastrointestinal disorders, ranging from diarrhea to constipation in humans (Kołodziej & Szajewska, 2019; Ojetti et al., 2014), as well as in reducing inflammation in necrotizing enterocolitis (NEC) and Treg-deficiency-induced autoimmunity in animal models (He et al., 2017; Liu, Tran, Fatheree, & Marc Rhoads, 2014), and generating beneficial metabolites and promoting immune surveillance in healthy newborns (Liu et al., 2019). However, its possible efficacy in ameliorating other organ functioning, such as liver, has not yet been explored. We therefore decided to evaluate the possible application of *Limosilactobacillus reuteri* DSM 17938 as a strategy to prevent WD-induced liver dysfunction. To this end, rats were fed a WD for 8 weeks and received probiotic *Limosilactobacillus reuteri* DSM 17938. The efficacy of the probiotic administration was investigated by assessing body composition, glucose homeostasis, hepatic insulin sensitivity, steatosis, inflammation, and mitochondrial function.

2. Methods

2.1. Animals and treatments

All experimental procedures involving animals were approved by "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University of Naples Federico II and authorized by Italian Health Ministry (137/2022-PR). This work complies with the animal ethics principles and regulations of the Italian Health Ministry. The authors ensured that all 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 ± 1 °C) with a 12 h light/dark cycle (06.30 – 18.30 h). The rats were divided in four groups (n = 8 for each group). The first group was euthanized at the beginning of the dietary treatment to measure initial body composition and energy balance (P0), the remaining three groups were fed a control diet (CD) or a high fat - high fructose diet alone (WD and WD-R). In addition, WD-R rats daily received 0.1 mL of a 10% sucrose solution containing 10^8 CFU of *L. reuteri* while C and WD 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 needless syringe and voluntarily consumed by rats. The probiotics were washed to remove the MRS broth before the administration. The diet treatment and probiotic administration were carried out for 8 weeks. The composition of the two diets is shown in Table S1.

During the treatment, body weight and food intake were monitored. At the end of the experimental period, the rats were anesthetized with sodium pentothal (40 mg kg^{-1} i.p.), euthanized by decapitation and livers were immediately collected. Liver samples were either freshly processed for mitochondrial assays or used to prepare paraffin embedded sections or snap frozen and stored for further analyses, as described below.

2.2. Cultivation of Limosilactobacillus reuteri DSM 17938

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

2.3. Quantification of fecal levels of Limosilactobacillus reuteri DSM 17938 by qPCR

Quantitative PCR (qPCR) was used to assess *L. reuteri* DSM 17938 levels in rat fecal samples at week 8. Primers LR1/1694f (5′-TTAAGGATGCAAACCCGAAC-3′) and LR1/1694r (5′-CCTTGTCACCTGGAACCACT-3′), targeting the gene encoding the cell surface protein Lr1694, were used to specifically detect *L. reuteri* DSM 17938. Samples were quantified for each single rat in 20 μL reactions containing 1x SYBR Green Master Mix (Thermo Scientific), 400 nmol/L of each primer and 2 μL of genomic DNA either non-diluted or diluted 1:5 in DNA-free milliQ water. qPCR was carried out under the following conditions: denaturation 95 °C, 5 min and 40 cycles of [95 °C, 10 s; 62 °C, 30 s] followed by melting curve analysis as previously reported by Rattanaprasert et al., 2014 (Rattanaprasert, Roos, Robert, & Hutkins, 2014).

Technical duplicates of biological replicates along with negative controls were examined. Standard curves were calculated using triplicates of tenfold serial dilutions of *L. reuteri* DSM 17938, ranging from 10^4 to 10^{11} copies. The target gene's copy number per microliter was calculated using the formula: (A B)/(C D); where A represents the DNA template's concentration in ng l^{-1}, B represents the Avogadro number (6.02×10^{23} copies mol^{-1}), C represents the average molecular weight of a DNA base pair (6.6×10^{11} ng/mol), and D represents the DNA size (bp).

2.4. Body composition and energy balance

Body composition was measured as previously described (Crescenzo et al., 2012). Briefly, the alimentary tract was cleaned of undigested food and the carcasses were then autoclaved. After dilution in distilled water and subsequent homogenization of the carcasses, duplicate samples of the homogenized carcass were analyzed to assess energy content by bomb calorimetry, body water content by drying method, body lipid content by the Folch extraction method, and body protein content using a formula relating total energy value of the carcass, energy derived from fat, and energy derived from protein, as detailed previously (Crescenzo et al., 2014). To take into account the energy content and composition of liver and gut, the same procedures used for homogenized carcasses were applied to tissue samples. Energy balance measurements were conducted by the comparative carcass technique over the experimental period (Crescenzo et al., 2013). Briefly, during the experimental period, metabolizable energy (ME) intake was determined by subtracting the energy measured in faeces and urine from the gross energy intake,

determined from daily food consumption and gross energy density of the diet. Body energy, fat and protein gain were calculated as the difference between the final and initial content of body energy, fat and protein, while energy expenditure was determined as the difference between ME intake and energy gain.

2.5. Glucose tolerance test

Blood samples were collected from a small tail clip in EDTA-coated tubes, both after 6 h of fasting and 15 and 30 min after intraperitoneal glucose injection (2 g kg^{-1}). Blood samples were centrifuged at 1400*g* for 8 min at 4 °C, plasma was collected and stored at − 20 °C for further determination of glucose and insulin. Plasma glucose concentration was measured by a colorimetric enzymatic method (GS Diagnostics SRL, Guidonia Montecelio, Rome, Italy). Plasma insulin concentration was measured using an ELISA kit (Mercodia AB, Uppsala, Sweden). Homeostasis Model Assessment (HOMA) Index was determined according to the formula: ((fasting glucose (mg/dl) × Fasting insulin (mU/l))/405) (Cacho, Sevillano, de Castro, Herrera, & Ramos, 2008). Area under the curve (AUC) for plasma glucose and insulin during the period 0–30 min of the glucose load was used for calculation of hepatic insulin resistance index (HIRI) according to the formula: (glucose (AUC) 0–30) × (insulin AUC 0–30) (Abdul-Ghani, Matsuda, Balas, & DeFronzo, 2007).

2.6. Metabolic parameters in plasma and liver

Colorimetric enzymatic methods were used to assess plasma metabolites by using commercial kits (SGM Italia, Rome, Italy for alanine aminotransferase (ALT), triglycerides, total cholesterol, high density lipoprotein (HDL)-Cholesterol).

Plasma lipopolysaccharide (LPS) levels were determined as previously reported (Mazzoli et al., 2021) using a kit based on a Limuls amaebocyte lysate (LAL) extract (ThermoFisher Scientific, Rockford, IL, USA). In brief, samples were mixed with the LAL reagent and chromogenic substrate reagent for 10 min, 37 °C. After exactly 10 min the Chromogenic Substrate solution was added for 16 min, 37 °C and absorbance readings were taken on a plate reader at 405 nm.

Plasma tumor necrosis factor alpha (TNFα), interleukin 6 (IL6) and interleukin 10 (IL10) concentrations were determined using a rat specific enzyme linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instruction.

Liver triglycerides were assessed as described above for plasma samples. Liver ceramides were assessed as described previously (Mazzoli et al., 2023). Hepatic steatosis was evaluated by hematoxylin and eosin staining of paraffin embedded sections. For the analysis, images were acquired with × 20 magnification, 3 random field/section per rat were analysed and scored blindly assigning a score between 0 (indicating absence of hepatic steatosis) and 5 (indicating the establishment of fatty liver disease) was assigned. The average of 3 random fields per animal was calculated, and the average of the 8 animal per group was used for the statistical analysis. Hepatic glycogen content was assessed by direct enzymatic procedure (Roehrig & Allred, 1974).

2.7. Hepatic mitochondrial function

Mitochondrial respiratory rates were assessed by High Resolution Respirometry (HRR) as reported previously (Mazzoli, Gatto, Crescenzo, Cigliano, & Iossa, 2021). Tissue samples were homogenized (1:1000, w/v) in Mir05 medium containing 110 mM sucrose, 60 mM K-lactobionate, 20 mM Hepes, 20 mM taurine, 10 mM KH2PO4, 6 mM MgCl2, 0.5 mM EGTA, 0.1% fatty acid free BSA, pH 7.0, and then aliquots of homogenates were transferred into calibrated Oxygraph-2 k (O2k, Oroboros Instruments, Innsbruck, Austria) 2 mL-chambers. Oxygen polarography was performed at 37 ± 0.001 °C (electronic Peltier regulation) in O2k -chambers and oxygen concentration (μM) as well as oxygen flux per tissue mass (pmol O2 s − 1•mg − 1) were recorded real-time using DatLab software (OROBOROS INSTRUMENTS, Innsbruck, Austria).

Oxygen consumption was firstly measured in the presence of malate (0.5 mM), pyruvate (5 mM) and glutamate (10 mM), to evaluate complex I-linked mitochondrial activity both before and after the addition of ADP 2.5 mM. Addition of succinate 10 mM was carried out to obtain complex I and II-linked respiration. Oligomycin at 2.5 μM was added to inhibit ATP synthase, followed by the addition of the uncoupler carbonylcyanide p-trifluoromethoxyphenyl-hydrazon (FCCP, 0.5 μM), to evaluate the maximal capacity of the electron transport chain. Rotenone (0.5 μM) was added to inhibit complex I and determine the maximal capacity supported by complex II alone. Residual oxygen consumption was established by 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. Lipid-supported respiration was measured in separate samples in the presence of palmitoyl carnitine 40 μM, malate 0.5 mM and ADP 2.5 mM. All samples were run in duplicates and the mean was used for analysis.

The stimulating effect of 10 mM exogenous cytochrome *c* on mitochondrial respiration in the presence of complex I-linked substrates and ADP was tested to evaluate mitochondrial integrity.

2.8. Oxidative stress parameters in liver

Homogenates from liver were prepared in 50 mM phosphate buffer, pH 7.0 (1:50 w/v) and used for the determination of oxidative stress markers.

Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid assay as previously described (Mazzoli et al., 2019).

Superoxide dismutase (SOD) activity was measured by following the decrease in the reduction rate of cytochrome *c* by superoxide radicals in a medium containing 50 mM KH_2PO_4 pH 7.8, 20 mM cytochrome *c*, 0.1 mM xanthine, and 0.01 units of xanthine oxidase, as previously reported (Mazzoli et al., 2021). Mitochondrial SOD was assayed following the same procedure in the presence of KCN.

Catalase activity was measured in 50 mM phosphate buffer, pH 7.0 containing 10 mM H_2O_2 and 0.25% Triton X-100 by monitoring the decomposition of H_2O_2 at 240 nm as described previously (Crescenzo et al., 2019).

Glutathione reductase (GR) activity was measured by following the decrease of NADPH absorbance at 340 nm as previously described (Mazzoli et al., 2023).

NADPH oxidase activity was assayed by monitoring the change in absorbance at 340 nM as previously reported (Mazzoli et al., 2019).

Xanthine oxidase activity was assessed by monitoring the production of uric acid at 290 nm as described previously (Mazzoli et al., 2021).

2.9. Western blot quantifications

Aliquots of protein extract (40 μg of proteins) were denatured in Laemmli's buffer (60 mM Tris pH 6.8, 10% sucrose, 2% SDS, 4% b-mercaptoethanol, 0.02% bromophenol blue) and loaded on a 10 % SDS–polyacrylamide gel. After the run, the gels were transferred on polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) at 0.8 mA/cm^2 for 90 min, as previously described (Cigliano et al., 2019). The membranes were preblocked in PBS, 3% bovine albumin serum, 0,3% Tween 20 for 1 h and then incubated overnight at 4 °C with antibodies against p-Akt (Cell Signaling, Danvers, MA, USA; diluted 1:1000 in blocking buffer), p-GSK (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:1000 in blocking buffer), toll-like receptor-4 (TLR4) (Sigma-Aldrich, St. Louis, MO, USA; 2 μg/mL in blocking buffer), phospho-nuclear factor k B (p-NFkB) (Santa Cruz biotechnology, Dallas, TX, USA; diluted 1:200 in blocking buffer), Oxphos (Abcam, Cambridge, United Kingdom.

; diluted 1:250 in blocking buffer), apolipoprotein E (APO-E) (Merk, Milan, Italy; diluted 1:500 in blocking buffer), low-density lipoprotein

receptor (LDL-R) (Abcam, Cambridge, United Kingdom; diluted 1:500 in blocking buffer), liver X receptor beta (LXR-β) (Santa Cruz biotechnology, Dallas, TX, USA; diluted 1:500 in blocking buffer), low density lipoprotein receptor-related protein 1 (LRP-1) (Santa Cruz biotechnology, Dallas, TX, USA; diluted 1:500 in blocking buffer). Membranes were washed and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA, diluted 1:5000 for p-Akt, p-GSK, TLR4, p-NFkB or 1:100000 for anti-Oxphos or 1:50000 for LDL-R, LXR-β, LRP-1 or 1:120000 for APO-E). The membranes were washed and incubated at room temperature with a chemiluminescent substrate, Immobilon (Millipore Corporation, Billerica, MA 01821, USA) for p-Akt, p-GSK, TLR4, p-NFkB, APO-E, LDL-R, LXR-β and LRP-1 or with the Excellent Chemiluminescent detection Kit (ElabScience, Microtech, Naples, Italy) for Oxphos. For loading control, membranes were stripped for 30 min at 50 °C in a solution containing Glycine 25 mM, SDS 1%, pH 2, incubated overnight at 4 °C with Akt polyclonal antibody (Cell Signaling, Danvers, MA, USA; diluted 1:1000 in blocking buffer) and used to normalize the p-Akt signal, GSK monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:1000 in blocking buffer) to normalize p-GSK signal, NFkB monoclonal antibody (Santa Cruz biotechnology, Dallas, TX, USA; diluted 1:200 in blocking buffer) to normalize p-NFkB signal, while actin was detected with polyclonal antibody (Sigma-Aldrich, St Louis, MO, USA; diluted 1:1000 in blocking buffer) and used to normalize the TLR4, OXPHOS, APO-E, LDL-R, LXR-β and LRP-1 signals. Quantitative densitometry of the bands was carried out by analyzing chemidoc or digital of X-ray films exposed to immunostained membranes. Quantification of signals was carried out by Image Lab

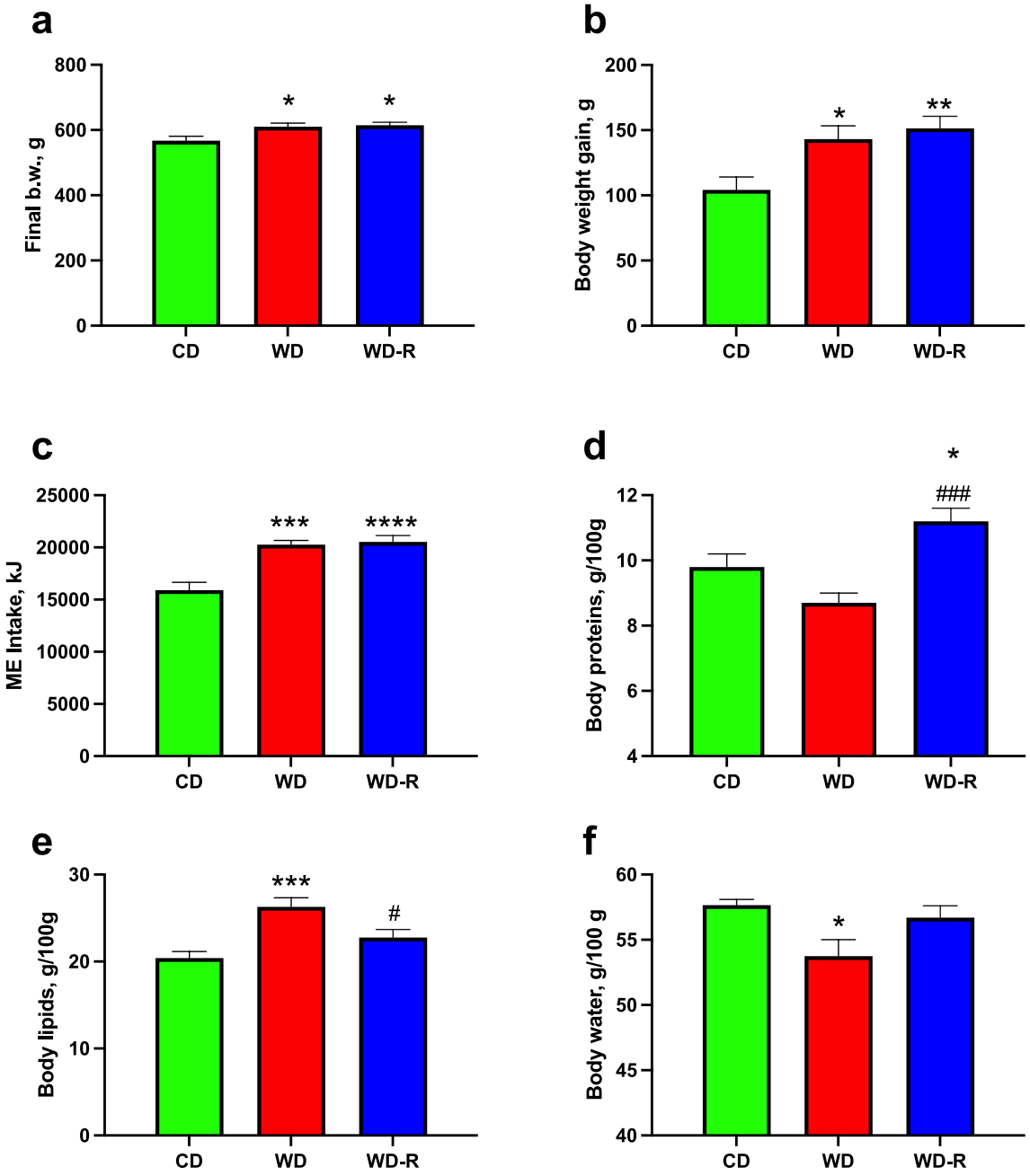

Fig. 1. Metabolic characteristics affected by WD and probiotic treatment. Final body weight (a), body weight gain (b), metabolizable energy (ME) intake (c), body proteins (d), body lipids (e) and body water (f) in CD, WD and WD-R rats. Values are the means ± SEM of 8 different rats. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to CD rats, #P < 0.05, ###P < 0.001 compared to WD rats (One way ANOVA followed by Bonferroni post-test).

Software (Biorad Laboratories S.r.L., Segrate (MI) – Italy).

2.10. Statistical analysis

Data were expressed as mean values ± SEM. The program GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA, https://www.graphpad.com) was used to verify that raw data have normal distribution and to perform One way ANOVA. A probability < 5% ($P < 0.05$) was considered statistically significant in all analyses.

3. Results

3.1. Metabolic characterization and plasma lipid profile

Metabolic characterization of our model evidenced that 8 weeks of western diet were able to induce changes in body composition typical of obesity onset. In fact, WD rats exhibited a significant increase in final body weight, body weight gain, ME intake and body lipids (Fig. 1a, b, c, d), and a decrease in body protein (Fig. 1d, not significant) and body water (Fig. 1 f, significant). Body energy, body energy gain, body lipid gain (Fig. 2a, b, d) were also significantly higher in WD rats, while body protein gain was significantly lower (Fig. 2c). Interestingly, supplementation with *L. reuteri* to rats fed with the WD, although not influencing body weight gain compared to WD rats, mitigated obesity development, so that WD-R rats exhibited lower body lipids and body lipid gain (Fig. 1e, 2d) and higher body protein and body protein gain (Fig. 1d, 2c) compared to WD rats.

In parallel with the establishment of obesity, WD rats exhibited significantly higher plasma levels of triglycerides (Fig. 3a), total cholesterol (Fig. 3b) and LDL cholesterol (Fig. 3d), with no changes in HDL cholesterol levels (Fig. 3c). Interestingly, the probiotic supplementation was beneficial for cholesterol homeostasis including normalization of plasma cholesterol (Fig. 3b) and plasma LDL (Fig. 3d), but not plasma triglycerides (Fig. 3a). In agreement, we found decreased hepatic levels of LDL receptors, LRP1, ApoE and LXR-β, involved in cholesterol homeostasis, in WD rats, while they were unchanged in WD-R rats compared to CD rats (Fig. 3e, f, g, h).

3.2. Systemic and hepatic inflammatory profile

WD administration elicited a condition of metabolic endotoxemia, characterized by increased plasma LPS levels (Fig. 4a). This in turn induced systemic inflammation, with increased plasma levels of proinflammatory cytokines TNFα and IL6 (Fig. 4b, c), as well as decreased levels of the anti-inflammatory cytokine IL10 (Fig. 4d). The above proinflammatory profile was partly reversed by probiotic administration (Fig. 4a, b, c). Metabolic endotoxemia and systemic inflammation affected inflammatory status in the liver, since WD induced an upregulation of TLR4 (Fig. 4e) and the activation of the pathway of NFkB (Fig. 4f).

3.3. Glucose homeostasis and insulin sensitivity

During the initial period after intraperitoneal glucose injection, it has been shown that muscle glucose uptake is minimally increased, while the time course of plasma glucose and insulin mainly reflects hepatic glucose handling (Brenner et al., 2017). Therefore, from the time course of plasma glucose and insulin from 0 to 30 min after glucose injection (Fig. 5a, b, c, d), it is possible to calculate HIRI. As reported in Fig. 5, HIRI was significantly higher in WD rats, while this increase was not detected in WD-R rats (Fig. 5f) and a similar pattern was also shown by HOMA index (Fig. 5e), thus delineating the establishment of a condition of insulin resistance in the liver elicited by WD but counteracted by probiotic administration. In agreement, we also found decreased

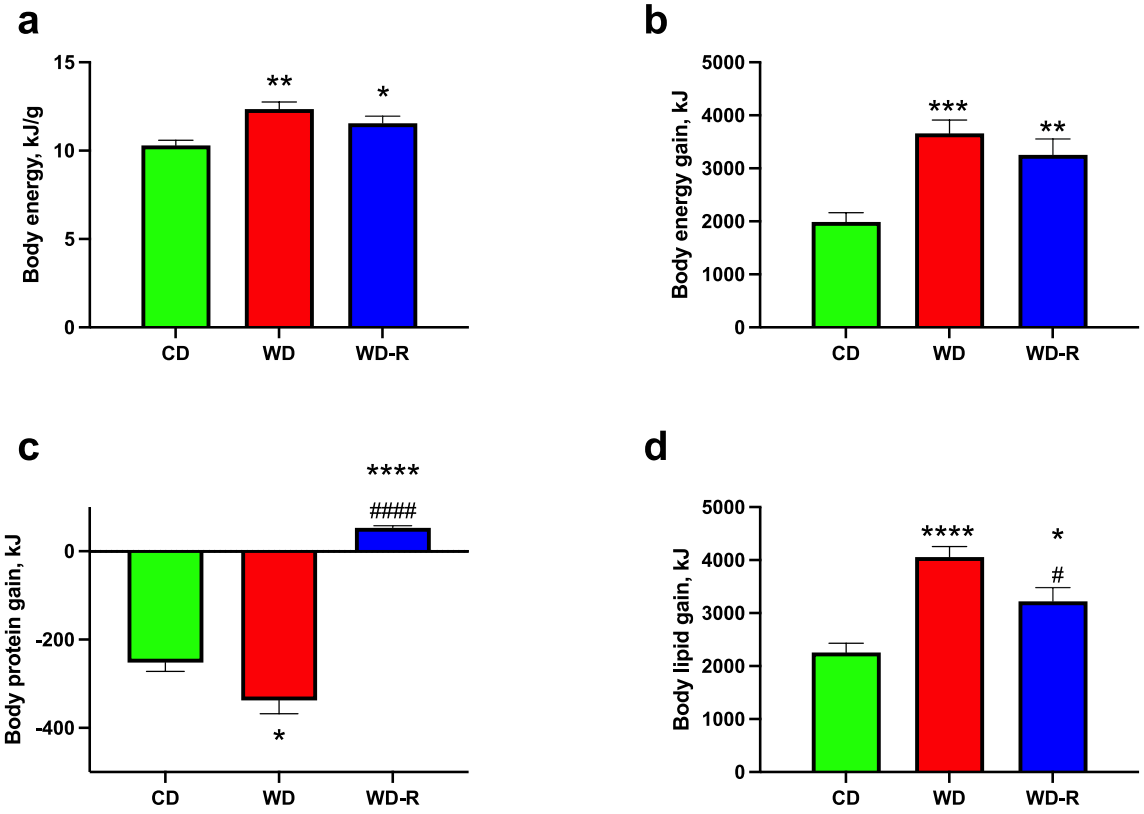

Fig. 2. Energy balance affected by WD and probiotic treatment. Body energy (a), body energy gain (b), body protein gain (c) and body lipid gain (d) in CD, WD and WD-R rats. Values are the means ± SEM of 8 different rats. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to CD rats, #P < 0.05, ####P < 0.0001 compared to WD rats (One way ANOVA followed by Bonferroni post-test).

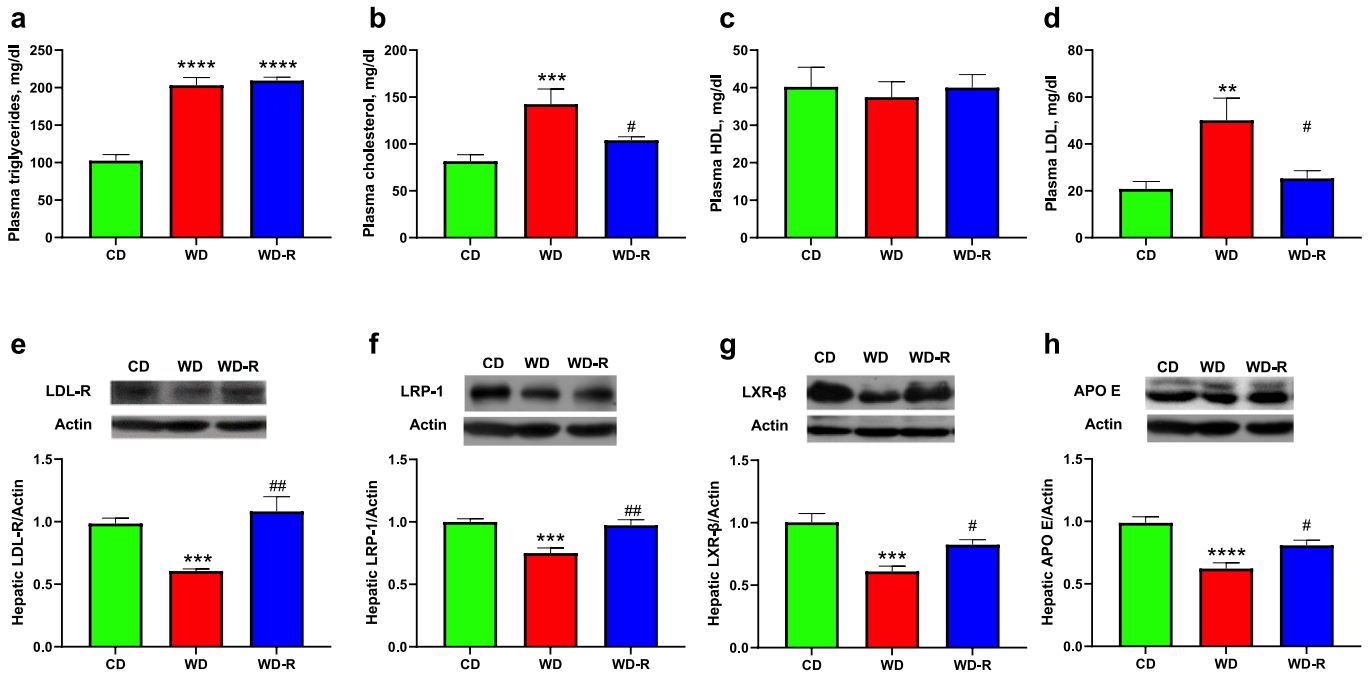

Fig. 3. Systemic metabolic parameters. Plasma triglycerides (a), total cholesterol (b), High Density Lipoprotein (HDL) cholesterol (c) and Low-Density Lipoprotein (LDL) cholesterol (d), hepatic levels of Low-Density Lipoprotein (LDL) receptor (e), LDL-receptor-related protein (LRP) 1 (f), Liver X receptor (LXR)-β (g) and Apolipoprotein E (ApoE) (h) (with representative western blot) in CD, WD and WD-R rats. Values are the means ± SEM of 8 different rats. **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to CD rats, #P < 0.05, ##P < 0.01 compared to WD rats (One way ANOVA followed by Bonferroni post-test).

phosphorylation of the downstream molecular effectors of insulin signaling in the liver, namely Akt and GSK (Fig. 5g, h), and a consequent decrease in hepatic deposition of glycogen (Fig. 5i) in WD but not WD-R rats.

3.4. Liver composition

Hepatic total lipids (Fig. 6a), triglycerides (Fig. 6b), ceramide (Fig. 6c) and degree of steatosis (Fig. 6d, e) were all significantly upregulated by WD, with a following increase in liver necrosis, as indicated by an increase in plasma ALT levels (Fig. 6f). Probiotic administration partially reversed TAG, steatosis, necrosis, and prevented ceramide accumulation (Fig. 6a-6f).

3.5. Mitochondrial physiology and oxidative stress

Mitochondrial functionality, assessed through HRR, evidenced a tendency to a decrease in WD rats with complex I-driven substrates, both in the absence (+PMG) and in the presence of ADP (+ADP), although it did not reach statistical significance (Fig. 7a). The WD-induced decrease was significant when respiration was also supported by complex II, in the presence of ADP (+succinate), following oligomycin addition (+oligomycin), and in the presence of complex I inhibitor (+rotenone) (Fig. 7a). Probiotic administration was effective not only in preventing this mitochondrial impairment but also in further stimulating mitochondrial oxidative capacity in the presence of succinate (+succinate; +oligomycin) and when complex I was inhibited by rotenone (+rotenone) (Fig. 7a). WD also induced a significant decrease in the rate of lipid-supported oxygen consumption, while *L. reuteri* administration was able to stimulate it also compared to C rats (Fig. 7b). In agreement with results shown in panel a, a significant decrease in the protein levels of complex II (Fig. 7f), assessed by Western blot, was found in WD rats, that was prevented by the probiotic, while no difference was detected in the amount of the other respiratory complexes (Fig. 7c, d, e, g).

Mitochondrial impairment is strictly linked to the onset of oxidative stress, even though other cellular sites are also involved in ROS production. Indeed, western diet also upregulated NADPH oxidase and xanthine oxidase activities (Fig. 8a, b), that represent additional sources of ROS. As a result, an increase in cellular oxidative stress can be inferred by the finding of higher levels of lipid peroxidation (Fig. 8c). The increased production of ROS was aggravated by the decrease in the antioxidant enzymes catalase (Fig. 8d) and GSR (Fig. 8e), while only SOD was upregulated as a counterregulatory response (Fig. 8f). Alle the above alterations were not detected in WD-R rats (Fig. 8).

4. Discussion

In this study, we tested the efficacy of the probiotic *Limosilactobacillus reuteri DSM 17938* against the deleterious metabolic effects induced by Western diet, that concur to the diagnosis of metabolic syndrome, namely obesity, insulin resistance, dyslipidemia, metabolic inflammation and fatty liver (Ambroselli et al., 2023).

Some recent publications have given support to a beneficial role of some strains of *L. reuteri* both in animal models and human studies. In fact, a beneficial role of *L. Reuteri* 99 was found in human studies on obesity, social behaviors, and neurodevelopment in Prader-Willi Syndrome (Kong et al., 2021), while several papers evidenced an antiobesity and lipid lowering efficacy in high-fat fed mice with *L. reuteri* MG5I49 (Choi et al., 2021); *L. reuteri* FGSZY33L6 (Zheng et al., 2021); *L. reuteri 6475* (Oh et al., 2020); *L. reuteri* NCUH064003 (Wei et al., 2023); *L. reuteri* DSM 32,910 (Larsen et al., 2023) and *L. reuteri* J1 (Zhang et al., 2022). Finally, Liu et al. (Liu et al., 2022) demonstrated that *L. reuteri* potentiated the antiobesity effect of caffeoylquinic acid in mice fed a high fat diet, although the probiotic alone was ineffective, and Rim Kim et al. (Kim et al., 2022) tested *L. reuteri* DSM 25175 in combination with other 2 probiotics.

In addition, there are also some indications on the potential benefit of *L. reuteri* DSM 17938, such as those reported by Jiang et al. (Jiang et al., 2021) about a protective role of this specific strain on liver failure, by Seif El-Din et al. (Seif El-Din et al., 2021), using DSM in combination with metformin and metronidazole to alleviate liver injury, by Fåk & Bäckhed (Fåk & Bäckhed, 2012), testing *L. reuteri* DSM in transgenic

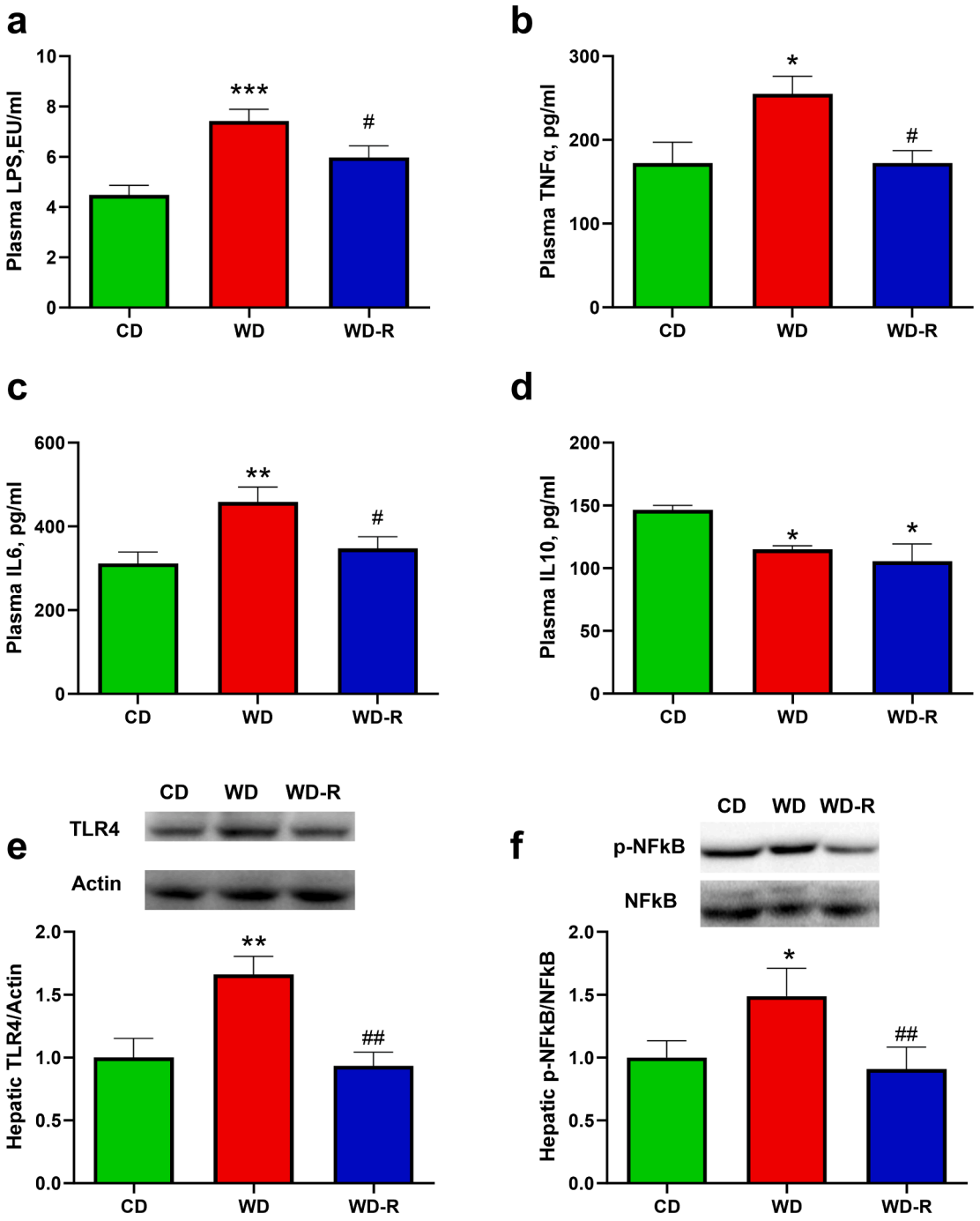

Fig. 4. Systemic and hepatic inflammation. Plasma Lipopolysaccharide (LPS) (a), plasma tumor necrosis factor alpha (TNFα) (b), plasma Interleukin 6 (IL6) (c) plasma Interleukin 10 (IL10) (d), hepatic protein content of toll-like receptor 4 (TLR4) (e), degree of phosphorylation of nuclear factor k B (NFkB) (f) (with representative blots, normalized to controls) in CD, WD and WD-R rats. Values are the means ± SEM of 8 different rats. *P < 0.05, **P < 0.01, ***P < 0.001 compared to CD rats, #P < 0.05, ##P < 0.01 compared to WD rats (One way ANOVA followed by Bonferroni post-test).

mice lacking ApoE, without evidencing any beneficial effect and Mobini et al. (Mobini et al., 2017), testing *L. reuteri* DSM 17938 in diabetic men and evidencing an increase in insulin sensitivity only in a subset of patients.

To our knowledge, this is the first study showing that this specific strain of *L. reuteri* proved to be useful not only in mitigating the development of obesity (in terms of increased body lipids) but more importantly in avoiding the decrease in body proteins, a metabolic change typical of aging and/or consumption of western diets. In parallel to this, our data evidenced that *L. reuteri* contrasted the onset of metabolic endotoxemia, inflammation and hypercholesterolemia and protected the liver from steatosis, inflammation and mitochondrial dysfunction.

WD is well known to induce obesity in adults, characterized by increased body weight and body lipids, due to increased energy intake, coupled to body protein loss due to both aging and decreased carbohydrate intake. Here we show that both WD and WD-R rats showed a higher ME intake with a consequent increase in body weight gain. The following increased lipid intake elicited by WD profoundly affected body composition, as demonstrated by increased body lipids in WD rats. Interestingly, *L. reuteri* administration contrasted the development of obesity in WD-R rats, with a lipid gain significantly lower compared to WD rats, notwithstanding the increased ME intake. More importantly,

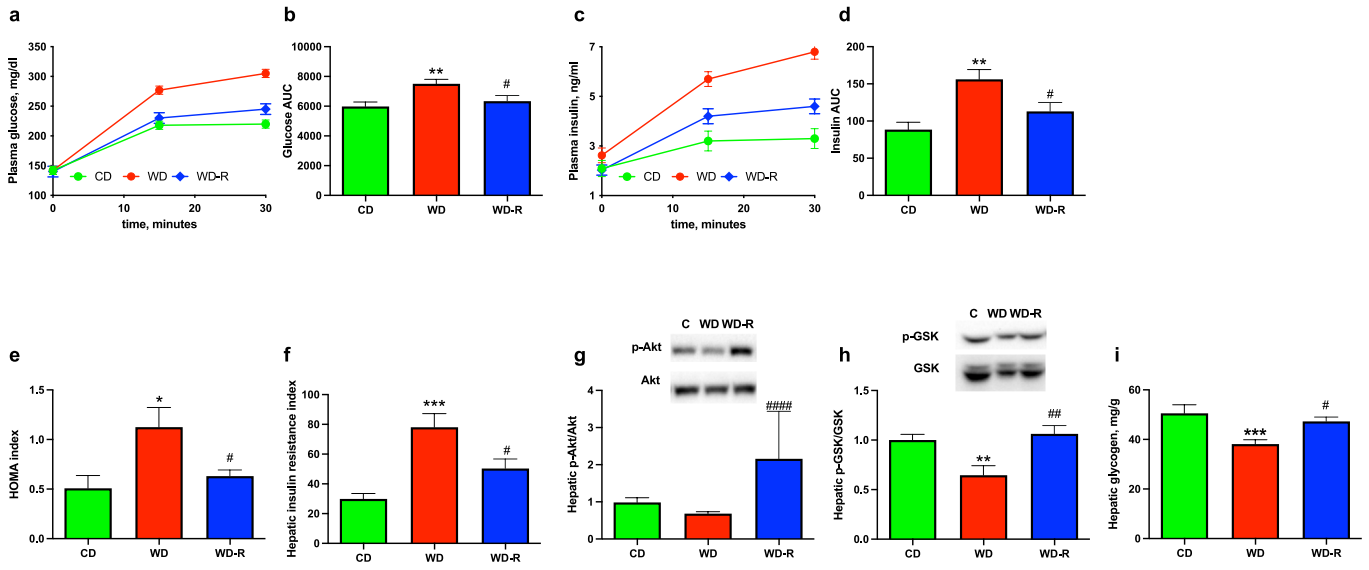

Fig. 5. Glucose homeostasis and hepatic insulin resistance. Plasma glucose and insulin during 30 min after intraperitoneal injection of glucose (a, c), glucose and insulin area under the curve (AUC) (b, d), Homeostatic Model assessment (HOMA) index (e), hepatic insulin resistance index (HIRI) (f), degree of phosphorylation of hepatic kinase Akt and Glycogen synthase kinase (GSK) (with representative western blot) (g, h) and hepatic glycogen levels (i) in CD, WD and WD-R rats. *P < 0.05, **P < 0.01, ***P < 0.001 compared to CD rats, #P < 0.05, ##P < 0.01, ####P < 0.001 compared to WD rats (One way ANOVA followed by Bonferroni post-test).

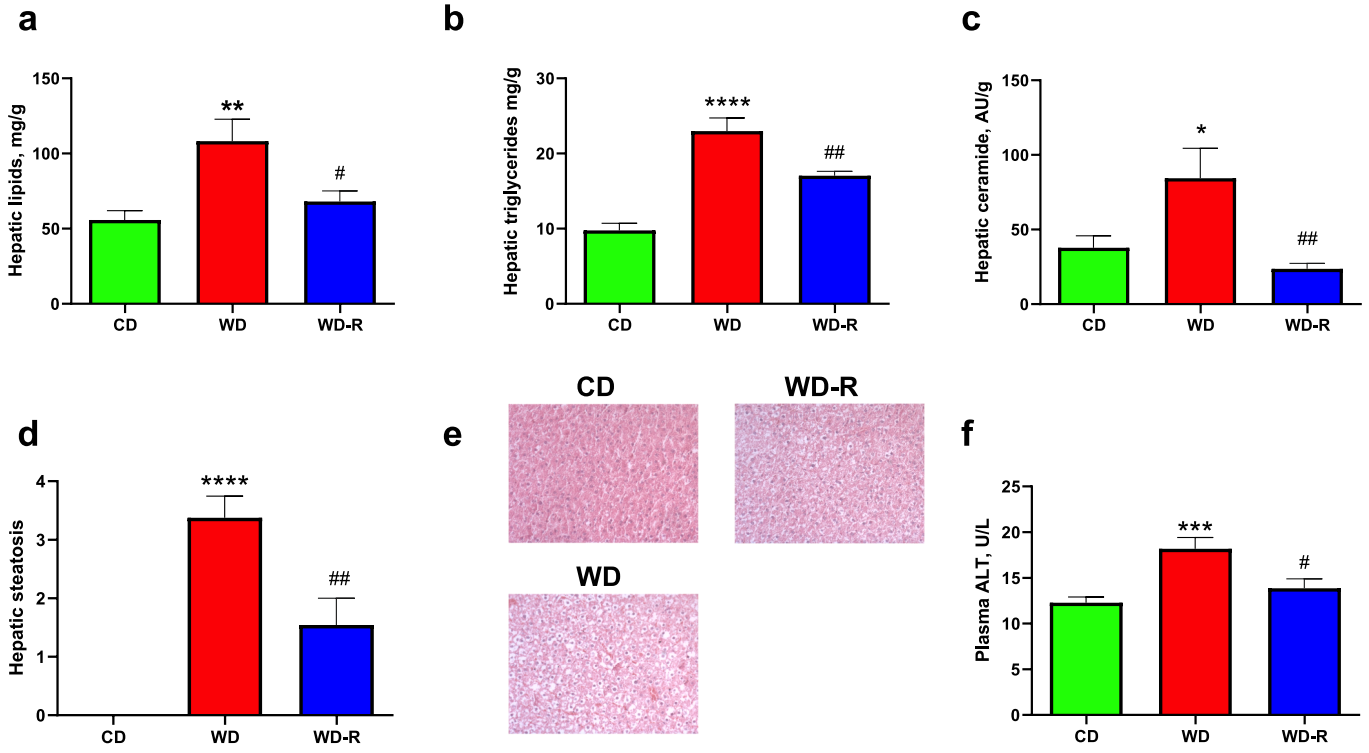

Fig. 6. Liver composition. Hepatic lipids (a), hepatic triglycerides (b), hepatic ceramide (c), hepatic steatosis (d, e) and plasma levels of alanine aminotransferase (ALT) (f) in CD, WD and WD-R rats. Values are the means ± SEM of 8 different rats. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to CD rats, #P < 0.05, ##P < 0.01 compared to WD rats (One way ANOVA followed by Bonferroni post-test).

the energy introduced and not stored as body lipids was diverted towards body proteins, so that WD-R rats displayed positive protein gain, instead of negative values as those found both in CD and WD rats. These results, if translated to humans that usually consume diets rich in fat and fructose, suggest that *L. reuteri* could be a useful strategy to counteract increased body lipids but more importantly decreased protein mass, that in turn impacts on the quality of life in the adult and more importantly in the elderly.

Notably, we also evidenced an important role of *L. reuteri* administration in handling another fundamental parameter that contributes to metabolic syndrome, namely cholesterol homeostasis, with a significant decrease in WD-R rats in plasma total cholesterol and LDL cholesterol levels, compared to WD rats, although the probiotic administration did not affect the increase in plasma triglyceride content induced by WD. The above results are in line with previous studies showing the hypocholesterolemic effect of other strains of *Lactobacillus* (Huang et al.,

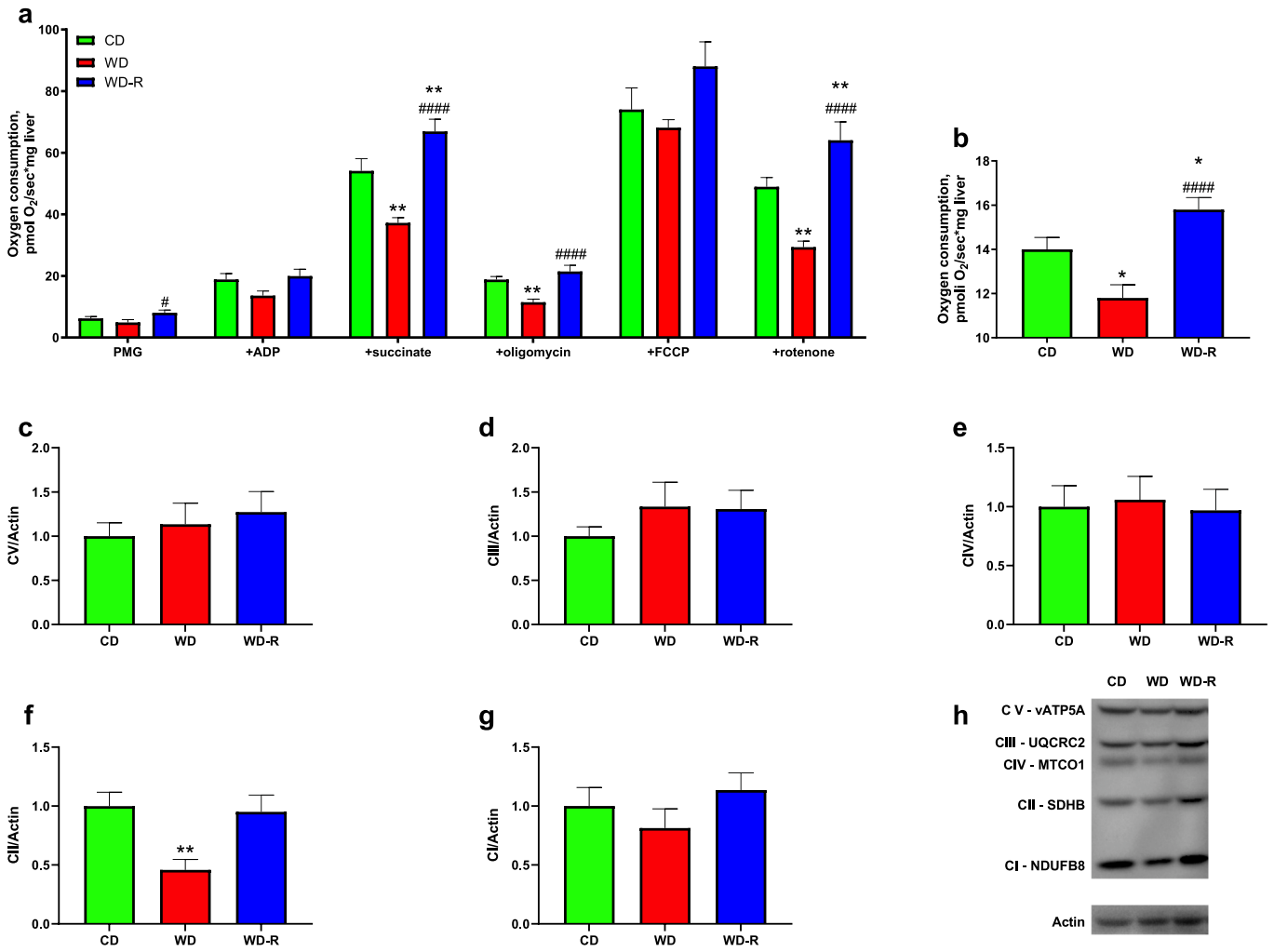

Fig. 7. Hepatic mitochondrial respiration. Hepatic respiratory rates (a), lipid-supported respiration (b), and protein content of the respiratory complexes (with representative western blot) (c, d, e, f, g, h) in CD, WD and WD-R rats. *P < 0.05, **P < 0.01 compared to CD rats, #P < 0.05, ####P < 0.0001 compared to WD rats (One way ANOVA followed by Bonferroni post-test).

2013; Khare & Gaur, 2020). We here report that the beneficial effect of *L. reuteri* on plasma cholesterol was at least partly due to reshaping, at molecular levels, of primary hepatic actors of cholesterol metabolism, namely LDL receptor, LRP1, ApoE and LXR-β (Spagnuolo et al., 2020), found downregulated in WD rats, but restored to control levels in WD-R rats. These latter findings point to an increased catabolism of LDL as a further index of the efficacy of the probiotic, since ApoE, by binding LDL-receptor and LRP-1, mediates cholesterol internalization in liver cells thus allowing cholesterol shuttling from peripheral cells to liver for excretion (Duan et al., 2022; Rasmussen, 2016).

Another index strictly linked to metabolic syndrome is chronic low-grade inflammation (Tarantino, Savastano, & Colao, 2010). Although signaling pathways and mechanisms underlying systemic inflammation have not been fully understood, several pieces of evidence have shown that the LPS–TLR4 signaling pathway plays a key role in systemic inflammation (Sun, Chen, Ren, & Li, 2022). Moreover, recently it has been shown the direct link between WD-driven alterations in gut microbiota with increased gut barrier permeability, referred to as the leaky gut syndrome (Mazzucca, Raineri, Cappellano, & Chiocchetti, 2021). In support of this picture, we observed an increase in the systemic plasma levels of LPS and of the two major inflammatory cytokines, TNFα and IL6, together with a decrease in the level of the anti-inflammatory cytokines IL10 in WD rats. All these parameters, exception made for IL10, were not altered in the rats fed with the WD diet that received *L. reuteri*. Several studies have reported the ability of *L. reuteri* administration to protect from the inflammatory bowel disease (IBD) by preventing the disruption of the intestinal barrier (Sengül, Işık, Aslım, Uçar, & Demirbağ, 2011; Shelby et al., 2022; Singh, Tehri, Kaur, & Malik, 2021). Considering that we show an enrichment of *L. reuteri* in the feces of WD-R rats compared to CD and WD (Fig. S1), it is conceivable that the probiotic presence is able to avoid the WD-induced decrease in gut barrier permeability, leading to a reduced passage of LPS, with a following reduction in the activation of the inflammatory signal cascade. On the other hand, it seems that *L. reuteri* DSM17938 is not able to regulate the expression of IL10, whose levels remain low due to the WD effect. The LPS-induced inflammation has a huge impact also at the level of the liver, one of the major organs involved in metabolism, as shown by an increase of hepatic levels of TLR4 and activation of NFkB in WD rats. In agreement with the beneficial effect of *L. reuteri* in preventing the establishment of systemic inflammation, both TLR4 and p-NFkB levels in WD-R rats were found comparable to the CD rats, confirming the potent anti-inflammatory effect exerted by the administration of this specific strain of *L. reuteri*.

Accumulating evidence suggests a role of hepatocyte-initiated inflammation in the development of insulin resistance (Jia et al., 2014). Indeed, the liver is a key insulin-responsive tissue and is actively involved in maintaining whole-body glucose and lipid metabolism. Therefore, as a primary consequence of WD ingestion and the subsequent systemic and hepatic inflammation here shown, a significant alteration of hepatic glucose homeostasis and insulin sensitivity was

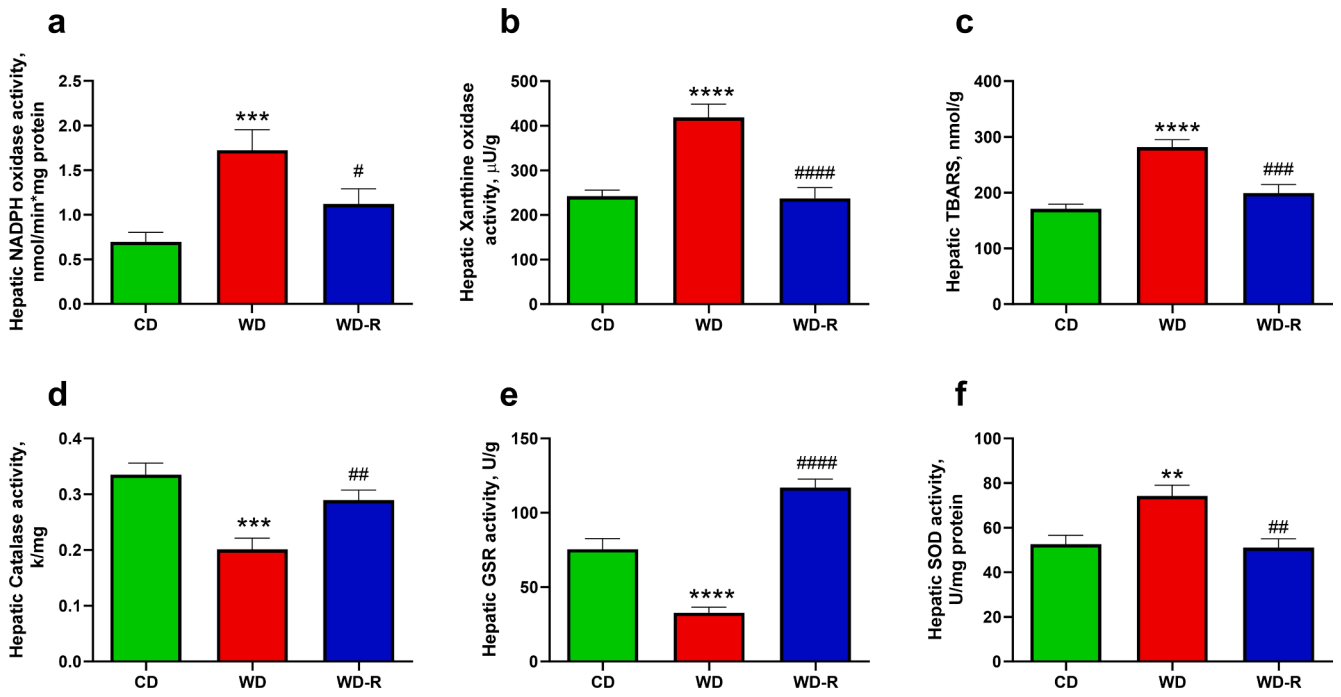

Fig. 8. Hepatic oxidative balance. Hepatic NADPH oxidase activity (a), xanthine oxidase activity (b), thiobarbituric acid reactive substances (TBARS) (c), catalase activity (d), glutathione reductase (GSR) activity (e) and superoxide dismutase (SOD) activity (f) in CD, WD and WD-R rats. **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to CD rats, #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 compared to WD rats (One way ANOVA followed by Bonferroni post-test).

evidenced, with the establishment of hepatic insulin resistance. In fact, WD rats showed increased HIRI and HOMA index, together with a decreased phosphorylation in the liver of two major protein of the insulin signaling cascade, namely p-Akt and p-GSK, with a following decrease in the hepatic level of glycogen. Given the ability of *L. reuteri* to protect from the development of inflammation, in WD rats supplemented with the probiotic, all these parameters in the liver showed levels comparable, if not ameliorated, when compared to CD rats, underlying the beneficial role of *L. reuteri* DSM 17938 in preventing alterations in glucose homeostasis and insulin sensitivity in the liver. Other *L. reuteri* strains have the ability to improve insulin resistance and ameliorate glucose levels in rats fed high fructose diet (Hsieh et al., 2013; Simon et al., 2015). Here we show the strong preventing effect of *L. reuteri* DSM 17938 against the WD-induced hepatic insulin resistance, but of particular note is the further increase in the level of p-Akt found in this study in WD-R compared to CD rats. This result can be explained considering that the PI3K/Akt is at the crossroads between several signalling pathways, playing a major role in the response to insulin, but also being involved in maintaining cell survival and inhibiting cell apoptosis, thus sustaining important cell functions in the process of stress response. In support of this speculation, it has been shown that *L. reuteri* was able to strongly increase PI3K/Akt phosphorylation in wound healing (Han et al., 2019).

Hepatic inflammation is one of the hits involved in the establishment of fatty liver disease, that however requires also an increased ectopic lipid deposition. In agreement, liver composition was also profoundly altered diet, as shown by increased level of triglycerides and plasma ALT, a critical marker of liver injury, in WD rats. Both parameters were unaltered in WD-R rats. The accumulation of triglycerides in the liver determines the development of hepatic steatosis, as displayed by WD rats and could further contribute to insulin resistance, since among the toxic intermediates of increased triglyceride synthesis we also detected an increase in liver ceramide, that interfere with the insulin signaling pathway (Sokolowska & Blachnio-Zabielska, 2019). Being able to prevent the condition of insulin resistance in the liver, *L. reuteri* DSM 17938 proves here also its ability to avoid the development of hepatic steatosis in WD-R rats. Among the underlying mechanisms of impaired hepatic lipid metabolism and increased hepatic steatosis, we identified a reduction in mitochondrial lipid-driven oxygen consumption. The involvement of mitochondria in the pathogenesis of insulin resistance has been implicated by many studies for more than a decade (Morino, Petersen, & Shulman, 2006; Rabøl et al., 2009; Vial, Dubouchaud, & Leverve, 2010). Similar findings have been reported in the liver of patients with nonalcoholic steatohepatitis (Pérez-Carreras et al., 2003), and this has relevance since the disease has been linked to hepatic insulin resistance and involve a condition of mitochondrial dysfunction (Sanyal et al., 2001).

The impaired lipid-linked oxidative capacity is the result of a decrease in complex II-driven respiration, that was also evident in uncoupled respiration after the addition of rotenone, a specific inhibitor of the flux from complex I to complex II, thus allowing us to measure only the flux through the respiratory chain from complex II onwards. The analysis of the various components of the mitochondrial respiratory chain in the liver revealed that the impairment of the complex II activity is due to a significant decrease in complex II amount, with no significant variation in the amount of the other complexes. Interestingly, in the rats that received *L. reuteri* these alterations in mitochondrial respiratory chain did not occur. Considering the above results, we can speculate that the protective effect exerted by *L. reuteri* on mitochondria is at the origin of the defense against the onset of insulin resistance and hepatic steatosis. This is supported by recent data showing that two species from *Lactobacillus genus* act upon liver mitochondria leading to the improvement of lipid metabolism (Rodrigues et al., 2021).

The development of the mitochondrial dysfunction and insulin resistance has frequently been suggested to involve increased production of reactive oxygen species (ROS) (Abdul-Ghani et al., 2009; Houstis, Rosen, & Lander, 2006) and it has been recently also suggested the existence of a link between mitochondrial dysfunction and other sources of ROS, such as NADPH oxidase (Dikalov, 2011) and xanthine oxidase (Gladden et al., 2011), which can generate a feed-forward vicious cycle of ROS production. Moreover, it has been suggested that complex II plays a role in ROS production under physiological and

pathophysiological conditions (Hadrava Vanova, Kraus, Neuzil, & Rohlena, 2020) since the functional loss of complex II can lead to succinate accumulation and ROS generation (Ralph, Moreno-Sánchez, Neuzil, & Rodríguez-Enríquez, 2011). In agreement, in our experimental model WD induced an increase in NADPH and xanthine oxidase activities, and consequently an increase of the oxidative damages to lipids in WD rats compared to CD. In parallel, WD rats also exhibited a decrease in the activity of the main antioxidant enzymes, namely catalase and GSR, while SOD activity resulted to be increased, probably as a counterregulatory response, determining in this way an alteration of the oxidative balance. In this case as well the administration of *L. reuteri* was efficient in preventing the oxidative stress to occur.

In conclusion, this study demonstrated that *Limosilactobacillus reuteri* DSM 17938 has a strong efficiency in preventing the development of the hepatic metabolic derangement elicited by the Western diet administration. In fact, the rats that received the WD and *L. reuteri* displayed a lower body lipid gain and a greater protein gain, underlying a beneficial effect of the probiotic in counteracting the development of obesity. Moreover, the WD-R rats were protected from the development of inflammation at a whole body and liver level and displayed normal glucose homeostasis and insulin sensitivity, a decreased hepatic lipid deposit and a normal function of the mitochondrial respiratory chain and redox balance (Fig. 9). These are promising results that open the way for the use of this probiotic as a prevention strategy against the development of diet-related diseases, such as type two diabetes and metabolic syndrome.

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Ethics Statement

All animal experiments were approved by the "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University of Naples Federico II and authorized by Italian Health Ministry (137/2022-PR)). This work complies with the animal ethics principles and regulations of the Italian Health Ministry. The authors ensured that all steps were taken to minimize the pain and suffering of the animals.

CRediT authorship contribution statement

AM designed the study. AM, ADP, VB, CG, RC, MSS, LC, ID and GM performed the experiments. LC, GM, SI and AM analyzed and graphed the data. AM and SI wrote the paper in consultation with LC. All authors contributed to data interpretation and performed final editing checks and approved the final manuscript.

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.

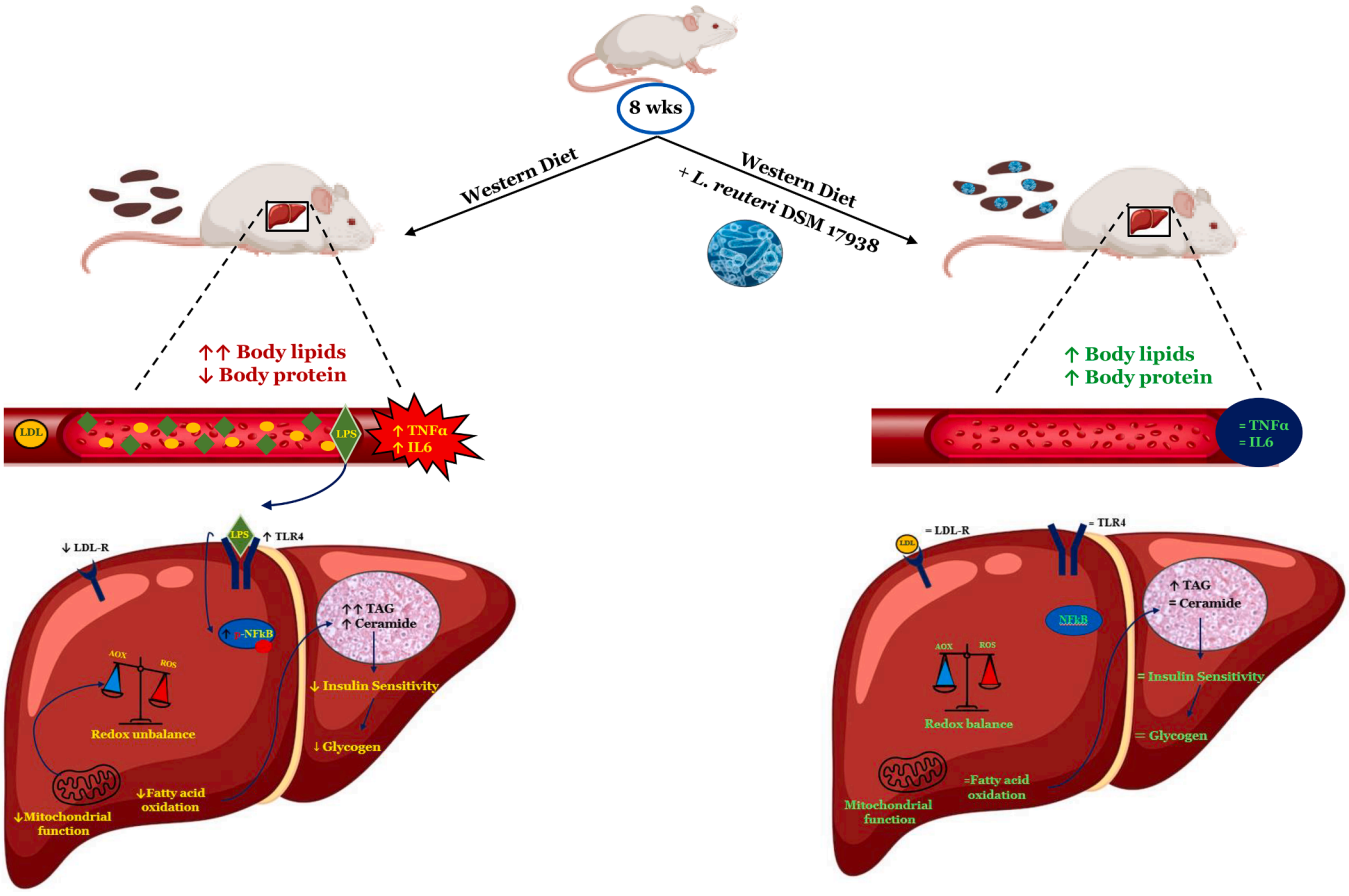

Fig. 9. Summary of changes in metabolic parameters elicited by western diet and counteracted by probiotic *Limosilactobacillus reuteri* DSM 17938. TNFα = tumour necrosis factor alpha; IL6 = interleukin 6; LDL-R = low density lipoprotein receptor; TLR4 = toll like receptor 4; LPS = lipopolysaccharide; p-NFkB = phosphonuclear factor k B; AOX = antioxidants; ROS = reactive oxygen species; TAG = triglycerides.

Data availability

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105805.

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