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RESEARCH PAPER

# Mitochondrial metabolism and neuroinflammation in the cerebral cortex and cortical synapses of rats: effect of milk intake through DNA methylation

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**Abstract**

Brain plasticity and cognitive functions are tightly influenced by foods or nutrients, which determine a metabolic modulation having a long-term effect on health, involving also epigenetic mechanisms. Breast milk or formula based on cow milk is the first food for human beings, who, throughout their lives, are then exposed to different types of milk.

We previously demonstrated that rats fed with milk derived from distinct species, with different compositions and nutritional properties, display selective modulation of systemic metabolic and inflammatory profiles through changes of mitochondrial functions and redox state in liver, skeletal and cardiac muscle. Here, in a rat model, we demonstrated that isoenergetic supplementation of milk from cow (CM), donkey (DM) or human (HM) impacts mitochondrial functions and redox state in the brain cortex and cortical synapses, affecting neuroinflammation and synaptic plasticity. Interestingly, we found that the administration of different milk modulates DNA methylation in rat brain cortex and consequently affects gene expression. Our results emphasize the importance of nutrition in brain and synapse physiology, and highlight the key role played in this context by mitochondria, nutrient-sensitive organelles able to orchestrate metabolic and inflammatory responses.



*Keywords:* Milk; DNA methylation; Gene expression; Neuroinflammation; Mitochondria dysfunction; Synapse.

## 1. Introduction

Milk is consumed by over 6 billion people around the world. People are exposed to different types of milk throughout their life. In the first six months, humans receive breast milk or formula based on cow milk with modified composition. Human milk (HM) provides an adequate supply of all nutrients necessary to support postnatal growth and development, as well as immunomodulatory elements essential for preventing metabolic and inflammatory illnesses throughout the whole life [1,2]. In particular, the first months of human life are a key period for brain growth, neuronal connectivity, cognitive development, behavioral and socio-emotional functions in adulthood. Therefore, proper brain nutrition during infancy significantly impacts one's entire life [3]. Later in life, the consumption of milk from other mammals is part of dietary recommendation in many countries, due to the milk's content of highly valuable proteins, fats, vitamins and minerals [4].

In previous studies, we investigated the effects of cow, human or donkey milk on glucose, lipid metabolism, oxidative stress and inflammatory parameters on organs and tissues with high metabolic rate, as liver, heart and skeletal muscle, with special emphasis on mitochondrial functions [5–7]. The brain is another tissue that requires a high level of energy to maintain its intricate architecture, as well as its flexibility and plasticity. Although it only makes up about 2% of total body weight, the brain has a high metabolic rate, using about 20% of the body's total ATP [8]. This

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high energy demand involves mitochondria, which are the powerhouse of the cells. Mitochondria are also related to inflammation and reactive oxygen species (ROS) production. As a result, mitochondrial dysfunctions, which cause oxidative stress and failure in satisfying the brain's high energy needs, are linked to neurological and neurodegenerative diseases [9]. The impaired mitochondria functions impact the brain much more than other organs [10]. The brain is particularly vulnerable to oxidative stress, primarily because of its high concentration of lipids that are vulnerable to oxidation, and its relatively low levels of antioxidants [11].

Notably, mitochondria located in synapses are critical in supplying energy to support synaptic functions. At presynaptic terminals, mitochondria produce most of the ATP required for exocytosis and neurotransmitter reuptake into synaptic vesicles. At the postsynaptic level, plasma membrane excitability, receptor functions, and ion channels functions also require significant energy [12]. Therefore, defects of synaptic mitochondria may result in synaptic failure, a common feature of neuropathologies [9]. Brain processes depend on synaptic plasticity, i.e., the great ability of the nervous system to change synaptic strength and neuronal connections in response to physiological stimuli and environmental changes. Long-term modulation of synaptic connections depends on ongoing gene expression changes. Interestingly, DNA methylation and chromatin remodeling play a role in controlling activity-dependent neuronal gene expression [13,14]. As a result, the activity-dependent dissociation of a repression complex made up of histone deacetylases and the protein MeCP2 with a methyl-CpG-binding domain occurs concurrently with the induction of brain-derived neurotrophic factor (BDNF) expression in cortical neurons [15]. In addition, in the long-term culture of cortical neurons under depolarizing conditions, it has been shown that a decrease in CpG methylation inside the BDNF promoter may be responsible for the prolonged increase of BDNF gene transcription [8,14]. These findings suggest that chromatin remodeling and DNA methylation can affect brain plasticity.

Based on these considerations, here we analysed, in the brain cortex and synaptosomal fraction of the rats, the effects of administration of human, cow and donkey milk on mitochondrial bioenergetics. In particular, we investigated if the milk-dependent mitochondrial modulation is related to epigenetic and gene expression changes in the cerebral cortex of the rats.

## 2. Materials and methods

Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cow milk (CM) was obtained from the "Nuova Latte Soc. Coop. Agr. A R.L." (Contrada Isca SNC, Eboli, SA, Italy). Donkey milk (from the Ragusana breed) (DM) was obtained from the "Az. Agric. Garofalo Patrizia" (Contrada Valle Cerasa, Casalbordino, CH, Italy). Human milk (HM) was kindly provided by the milk bank of the Macedonio Melloni Hospital (Department of Childhood and Evolutionary Age Medicine, Milano, Italy). A standard rodent diet (4RF21) was purchased from Mucedola (Mucedola srl, Settimo Milanese, MI, Italy).

### 2.1. Animal, diet, and tissue collection

Male Wistar rats (60 days old; 345 ± 7 g; Charles River, Calco, Lecco, Italy) were individually caged in a temperature-controlled room and exposed to a daily light–dark cycle (12/12 h) with free access to chow standard rodent diet (15.88 kJ/g) and drinking water. The rats were divided into four experimental groups ($n = 7$). Three of them received a 4-week supplementation with equicaloric intake (82 kJ/day) of raw CM, DM, or HM drinking 21, 48, or 22 ml/day, respectively, according to the experimental plan previously reported (Supplementary Tables 1, 2) [16]. The dose was selected considering that milk is administered as complementary food of the diet, and that the administered amount must be completely drunk each day by the animals, without affecting the daily intake of the standard diet. The volume of each type of milk varies according to its energy density. HM was obtained from the human milk bank before the heat treatment. The last group did not receive milk supplements and was used as a control.

The gross energy density for the standard diet, CM, DM, or HM (15.8, 14.04, 13.79 or 14.01 kJ/g, respectively) were determined by bomb calorimeter (Parr adiabatic calorimeter, Parr Instrument Co., Mo-line, IL, USA). At the end of the treatments, the animals were anesthetized by intra-peritoneal injection of chloral hydrate (40 mg/100 g body weight), and blood was taken from the inferior cava. Blood was taken from the inferior cava, and serum was obtained by centrifuging at 1,000 × g for 10 min and stored at −80°C for subsequent biochemical analyses. The cerebral cortex was removed and subdivided; samples not immediately used for synaptosomes, and mitochondria preparation were frozen and stored at −80°C for subsequent determinations.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, enforced by Italian D.L. 26/2014, and approved by the animal care and the Committee of the University of Naples Federico II (OPBA), Naples, Italy and the Italian Ministry of Health, Rome, Italy (authorization n. 97/2019-PR).

### 2.2. Preparation of mitochondria and synaptosomes from the cerebral cortex

Synaptosomes were prepared using the standard procedure [17], yielding a well-characterized synaptosomal fraction [18,19]. In brief, the cerebral cortex was quickly dissected and homogenized in a Dounce homogenizer with nine volumes of cold isotonic medium (IM) containing 0.32 M sucrose and 10 mM Tris-Cl, pH 7.4. To prepare subcellular fractions, following centrifugation of the homogenate (2,200 g, 1 min, 4°C), the sediment was resuspended in the same volume of IM and centrifuged under the same conditions to yield sediment containing nuclei, cell debris, and large synaptosomes (P1). The mixed supernatant fractions were centrifuged at higher speed (23,000 g, 4 min, 4°C) to yield a sediment that was resuspended in IM and centrifuged under the same conditions to produce a second sediment containing free mitochondria, synaptosomes and myelin fragments (P2) that was resuspended in IM. Differential centrifugation of P2 aliquots was used to prepare isolated synaptosomes and mitochondria. Further purification of synaptosomes was achieved by fractionating an aliquot of the P2 fraction on a discontinuous Ficoll gradient. One milliliter of the P2 fraction brought to a final protein concentration of 3.5 mg/ml, was layered over a discontinuous gradient of 5% and 13% Ficoll dissolved in IM (2 ml each), and centrifuged at 45,000 g, for 45 min, 4°C. The purified synaptosomal fraction was recovered at the interface between the two Ficoll layers, diluted with nine volumes of IM, and sedimented by centrifugation (23,000 g, 20 min, 4°C). The sediment was homogenized in IM, and protein concentration was determined by Bradford colorimetric assay (Biorad) using bovine serum albumin (BSA) as standard. To obtain mitochondrial fraction, the P2 fraction was resuspended in a medium containing 80 mM LiCl, 50 mM HEPES, 5 mM Tris-PO4, 1 mM EGTA and 0.1% (w/v) fatty-acid-free BSA, pH 7.0, and centrifuged at 500 g, 10 min, 4°C. The supernatant was centrifuged at 10,000 g for 10 min at 4°C; the pellet was washed once and resuspended in a medium containing 80 mM LiCl, 50 mM HEPES, 5 mM Tris-PO4, 1 mM EGTA and 0.1% (w/v) fatty-acid-free BSA, pH 7.0. The protein content of the mito-

chondrial fraction was determined by Bradford colorimetric assay (Biorad) using BSA as standard. The quality of isolated mitochondria was assured by checking that contamination of mitochondria by other ATPase-containing membranes was lower than 10%. That addition of cytochrome c (3 nmol/mg protein) enhanced only state 3 respiration by approximately 10% [20].

### 2.3. Measurements of mitochondrial oxidative capacities and degree of coupling

High-resolution respirometry Hansatech oxygraph (Yellow Spring Instruments, Yellow Springs, OH, USA) was used to assess the oxygen consumption rate in mitochondria isolated from the cerebral cortexes. Isolated mitochondria were incubated at a temperature of 30°C in a medium (pH 7.0) containing 80 mM KCl, 50 mM HEPES, 5 mM $KH_2PO_4$, 1 mM EGTA and 0.1% (w/v) fatty-acid-free BSA to oxidize their endogenous substrates for a few minutes. Then, different substrates were added at the following concentrations: 10 mM succinate plus 3.75 mM rotenone; 40 μM palmitoyl-L-carnitine plus 2.5 mM malate. State 4 oxygen consumption was obtained in the absence of ADP, and State 3 oxygen consumption was measured in the presence of 0.3 mM ADP. The respiratory control ratio (RCR) was calculated as the ratio between states 3 and 4, according to Estabrook (Estabrook, 1967). The degree of coupling was determined in the brain by applying an equation by Cairns et al. [21]: degree of coupling $= \sqrt{1-(Jo)sh/(Jo)unc}$

Where $(Jo)sh$ represents the oxygen consumption rate (OCR) in the presence of oligomycin that inhibits ATP synthase, and $(Jo)unc$ is the uncoupled rate of oxygen consumption induced by carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), which dissipates the transmitochondrial proton gradient. (Jo)sh and (Jo)unc were measured as above using succinate (10 mmol/L) and rotenone (3.75 μmol/L) in the presence or absence of palmitate (15 μM) and after the addition of oligomycin (2 μg/ml) or FCCP (1 μmol/L), respectively. Carnitine-palmitoyl-transferase (CPT) activity was followed spectrophotometrically as CoA-sH production using 5,50 - dithiobis (nitrobenzoic acid) (DTNB) and as substrate palmitoyl-Coa 10 μM. The medium consisted of 50 mM KCl, 10 mM Hepes (pH 7.4), 0.025% Triton X-100, 0.3 mM DTNB, and 10–100 pg of mitochondrial protein in a final volume of 1.0 mL. The reaction was followed at 412 nm at 25 °C in a thermostated spectrophotometer and enzyme activity was calculated from an E412 = 13,600/(M × cm) [22]. The rate of mitochondrial hydrogen peroxide ($H_2O_2$) release was assayed by following the linear increase in fluorescence (excitation 312 nm and emission 420 nm) due to the oxidation of homovanillic acid in the presence of horseradish peroxidase [23]. Superoxide dismutase (SOD) specific activity was measured in a medium containing 0.1 mM EDTA, 2 mM KCN, 50 m $KH_2PO_4$, pH 7.8, 20 mM cytochrome c, 5 m xanthine, and 0.01 U of xanthine oxidase. Enzyme activity was measured spectrophotometrically (550 nm) at 25 °C, by monitoring the decrease in the reduction rate of cytochrome c by superoxide radicals, generated by the xanthine-xanthine oxidase system. One unit of SOD activity is defined as the concentration of enzyme that inhibits cytochrome c reduction by 50% in the presence of xanthine and xanthine oxidase [24].

### 2.4. Seahorse XFp analyzer Cell Mito Stress Test

Oxygen consumption (OCR) and extracellular acidification rate (ECAR) measurements in synaptosomal fraction from rat cortex were performed by Seahorse XFp analyzer (Seahorse Biosciences, North Billerica, MA, USA), by using Cell Mito Stress Test kit (Seahorse Bioscience, 101706-100). XFp cartridges (Seahorse Bioscience) were hydrated by incubation with 200 μl calibrant (Seahorse Bioscience) per well at 37°C overnight. Before loading a cartridge onto the XFp analyzer, 20 μl of 30 μM oligomycin, 22 μl of 40 μM FCCP, 24 μl of 20 μM rotenone/antimycin A were added to the cartridges (final concentrations of oligomycin, FCCP, and rotenone injected during the assay were 3 μM, 4 μM, and 2.0 μM, respectively). The cartridge was loaded onto the XFp analyzer for calibration. In each well of Seahorse XFp plate (Seahorse Bioscience, North Bilerica, MA, USA), precoated with poly-D-lysine, we seeded 10 μg of synaptosomal protein in a final volume of 100 μl ionic medium (20 mM HEPES, 10 mM D-Glucose, 1.2 mM $Na_2HPO_4$, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, 5 mM KCl, 140 mM NaCl, pH 7.4 at 4°C). The plate was centrifuged at 2,000 g for 1 h at 4°C in a swinging bucket rotor (Thermo Scientific 75003624) in a Thermo Fisher Scientific 3000R centrifuge. Before Cell Mito Stress analyses, the medium was replaced with 180μl of incubation medium (3.5 mM KCl, 120 mM NaCl, 1.3 mM $CaCl_2$, 0.4 mM $KH_2PO_4$, 1.2 mM $Na_2SO_4$, 2 mM $MgSO_4$, 4 mg/ml BSA, 15 mM D-glucose, 5 mM pyruvate, 2.5 mM malate, pH 7.4 at 37°C). The basal OCR was determined in the presence of the incubation medium. After 20 minutes, the oligomycin, an ATP synthase inhibitor, was added to calculate cells' oxygen consumption to synthesize ATP (ATP-linked respiration) and oxygen consumption used to overcome the proton leak across the mitochondrial membrane. Afterward, the mitochondrial electron transport chain was stimulated maximally by the addition of the uncoupler FCCP. FCCP treatment collapses the proton gradient and disrupts the mitochondrial membrane potential, which allows measurement of the maximal uncoupled respiration (maximal respiration). Finally, a combined treatment of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor, was used to shut down mitochondrial respiration, which enables differentiation between the mitochondrial (basal respiration) and non-mitochondrial respiration contribution to total respiration. Coupling efficiency is the proportion of the oxygen consumed to drive ATP synthesis compared with that driving proton leak and was calculated as the fraction of basal mitochondrial OCR used for ATP synthesis (ATP-linked OCR/basal OCR) [25].

### 2.5. Brain and synaptosomes parameters

The malondialdehyde (MDA) level was measured using the thiobarbituric acid (TBAR) method to determine the lipid peroxidation in cerebral cortex homogenate and synaptosomal fraction. MDA reacts with thiobarbituric acid (TBA) to form a pink chromogen that is detected at the wavelength of 532 nm. MDA values were expressed as micromoles per milligram of protein [26]. The levels of ROS were determined as previously reported [27,28]. An appropriate volume of freshly prepared tissue homogenate was diluted in 100 mM potassium phosphate buffer (pH 7.4) and incubated with a final concentration of 5 μM dichlorofluorescein diacetate (Sigma-Aldrich) in dimethyl sulfoxide for 15 min at 37°C. The dye-loaded samples were centrifuged at 12 500 × g for 10 min at 4°C. The pellet was mixed at ice-cold temperatures in 5 mL of 100 mM potassium phosphate buffer (pH 7.4) and incubated for 60 min at 37°C. The fluorescence measurements were performed with an HTS-7000 Plus plate reader spectrofluorometer (Perkin Elmer, Wellesley, MA, USA) at 488 nm for excitation and 525 nm for emission wavelengths. ROS were quantified from the dichlorofluorescein standard curve in dimethyl sulfoxide (0–1 mM). Reduced (GSH) and oxidized glutathione (GSSG) concentration in the cerebral cortex homogenate and the synaptosomal fraction were measured with the dithionitrobenzoic acid-GSSG reductase recycling assay; the GSH to GSSG ratio was used as an oxidative stress marker (Viggiano et al., 2016). The levels of tumor necrosis factor-α (TNF-

$\alpha$), interleukin-1$\beta$ (IL-1$\beta$), interleukin-6 (IL-6) and interleukin-10 (IL-10) in the cerebral cortex homogenate and in synaptosomal fraction were determined as previously reported [29], by using commercially available ELISA kits to determine serum interleukin-1$\alpha$ (IL-1$\beta$), interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) (Thermo Scientific, Rockford, IL, USA; Biovendor R and D, Brno, Czech Republic).

### 2.6. RNA and DNA extraction

Genomic DNA and total RNA were extracted from rat brain tissue from different diets. Briefly, each tissue was homogenized in 50 μl for each mg of the following buffer: 4M guanidine isothiocyanate, 25 mM sodium citrate pH 7.00, 0,5% N-lauroyl-sarcosine, 0,1M $\beta$-mercaptoethanol. For the DNA extract, was added TE 2/3 V, 1% SDS, and 200 μg/ml of Proteinase K, and incubated at 37°C overnight. The following day, DNA was purified once with 1V of Phenol/Chloroform/Isoamyl Alcohol, 25:24:1 (v/v), and then with 1V of chloroform. Following manufacturer protocol, the DNA was treated with RNase A (10109142001 from Merck); ethanol precipitated, dried, and resuspended in TE buffer. For RNA isolation, 1V acid phenol pH 4, 1/10V sodium acetate 2M pH 4,2 and 0,2V chloroform were used, followed by isopropanol precipitation. Finally, 1 μg of DNA and RNA, DNAase free (#04716728 Roche), was sent to IGA Technology Services for Reduced-representation bisulfite sequencing (RRBS-Seq) and RNASeq sequencing under company instructions.

### 2.7. Reduced representation bisulfite sequencing (RRBS-Seq)

200-ng genomic DNA from the 12 samples (only males, 3 for group) was digested with MspI (NEB). Ovation RRBS Methyl-Seq (NuGEN, Redwood City, CA) has been used for library preparation after the digested products were subjected to end-repair, A-tailing, and adapter-ligated use a TruSeq preparations kit (Illumina) under the manufacturer's instructions. The raw reads were cleaned for quality and adapters using in-house tools removing TrueSeq adapters and clipping read tails of Phred quality less than 20 processed following Ovation RRBS pipeline (https://github.com/nugentechnologies/NuMetRRBS) using NuGEN DuplicateMarking tool, NuDup (nugentechnologies.github.io/nudup/) to discriminate between true PCR duplicates and independent adaptor ligation events to fragments with the same start site. The reads were mapped on the rat genome version rn5 using Bismarkbismark v 0.22.3 [30] with standard parameters. Methylation values were extracted and saved as .cov files using *bismark_methylation_extractor*. CpG methylation values were then analyzed using Metilene v 0.2–7, requiringRnBeads, CpG sites with a coverage of less than 10 reads in at more than 50% of samples or more than 10 times the 95-percentile of coverage be excluded from subsequent analyses.

### 2.8. RNA sequencing

RNAseq libraries from 12 samples (only males, 3 for group) were prepared using the TruSeq Stranded mRNA kit (Illumina, San Diego, CA) according to the manufacturer's instructions (Library type: fr-firstrand). Adapter sequences were masked with Cutadapt v1.11 from raw fastq data using the following parameters: –anywhere (on both adapter sequences) –overlap 5 –times 2 –minimum-length 35 –mask-adapter. Raw reads processing, mRNA levels estimation and differential expression analysis was performed using ARMOR pipeline v 1.4.0 [31]. The raw reads were cleaned for quality and adapters using trimGalore (https://github.com/FelixKrueger/TrimGalore) removing TrueSeq adapters and clipping read tails of phred quality less than 20. The reads were mapped to the rn5 transcriptome using SALMON v 1.0.0 [31]. Transcript quantifications from Salmon were then exported using tximeta [32], and subsequent differential expression analysis was performed with edgeR [33].

### 2.9. Pathway analysis

Rattus Novegicus annotation was used to perform KEGG pathway analysis [34] by transferring the gene abundances via the distinctive gene symbols and names. We only accepted *P*-values with a Bonferroni corrected value of less than .05 when looking for a significant overrepresentation of KEGG pathways [35].

### 2.10. Real-time quantitative PCR (qPCR)

cDNA was synthesized in 20-μl reactions containing 1 μg of total RNA, 100 U of Superscript III Reverse Transcriptase (Invitrogen), and 2 μl random hexamer (20 ng/μl) (Invitrogen). mRNA was reverse-transcribed for 1 h at 50°C; the reaction was then heat-inactivated for 15 min at 70 °C. The products were stored at −20 °C until use. qRT-PCR was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems) using the SYBR Green-detection system (FS Universal SYBR Green Master Rox/Roche Applied Science) with cycle conditions as follows: 95°C 10′; 35x (95°C 15′′, 60°C 90′′); 95°C 15′′, 60°C 1′, 95°C 15′′); 72°C 10 min. Each sample was assayed in triplicate. All reactions were normalized to 18S mRNA. The complete list of oligonucleotides used is reported in Table 1. All data are presented as means ± SEM. Data were analyzed using JMP Statistical DiscoveryTM software 6.03 by SAS (Statistical Analysis Software) and tested for normal distribution of variables using the Shapiro-Wilks test ("normal distribution fit" tool—JMP software). One-way analysis of variance (ANOVA) has been used to compare groups. Then, validation of selected genes for each group versus control was determined using Student's t-test. Asterisks indicate significance at $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ throughout.

### 2.11. Western blot

Aliquots of cerebral cortex homogenate were denatured at 100°C for 5min in sample buffer (60 mM Tris-HCl pH 6.8, 10% Glycerol, 2% SDS, 100 mM DTT 0.1% bromophenol blue), separated in 10–15% SDS-PAGE and transferred to PVDF membranes (Merck-Millipore). Western blot analysis was performed as previously reported [19], probing the membrane with the following antibodies: synaptophysin (1:1.000.000, AB9272 Millipore), syntaxin (1:30.000, E-AB-33012 Elabscience) and $\beta$-actin (1:2.000, 612656 BD Biosciences). Secondary antibody, linked to horseradish peroxidase, was against rabbit (1:20.000, A0545, Sigma-Aldrich) or mouse (1:20.000, NA931, GE Healthcare). Signals were visualized by chemiluminescence (ECL, Millipore) on X-ray film (Fujifilm X-Ray Film).

### 2.12. Ethics statement

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, enforced by Italian D.L. 26/2014, and approved by the animal care and the Committee of the University of Naples Federico II (OPBA), Naples, Italy and the Italian Ministry of Health, Rome, Italy (authorization n. 97/2019-PR).

Table 1
List of DNA oligonucleotides used for real-time quantitative PCR (qPCR).

| Gene | FW | Rev |
|---|---|---|
| Rab18 | 5'-CAGACGACACCTTTGATCC -3' | 5'- TCCCATATTGCAAGTTTAGCC -3' |
| Chrm4 | 5'- GCAGAGAAAGACAGAAGCAG -3' | 5'- CATTGACAGGCGTGTGAAGTTG -3' |
| Timm22 | 5'- AGGAGCAGAAGATGATCGAG -3' | 5'- TCAATGCCAGCAGTAAAGAC -3' |
| Cdhr1 | 5'- CTTCCGCGGAGACATGAGG -3' | 5'- AGAAGTGAGGGGCAAAGTTGG- 3' |
| Slc17a6 | 5'- TTTGCTGGAAAATCCCTCGG -3' | 5'- AGCACGTACAGTCGCATAGC -3' |
| 18S | 5' - CATTCGAACGTCTGCCCTAT - 3' | 5' – GTTTCTCAGGCTCCCTCTCC – 3' |

The gene identification tag (ID) is shown on the left, the forward primer sequence (FW) is in the center, and the reverse primer sequence (Rev) is on the right.

### 2.13. Statistical analysis

All data are presented as means ± SEM. Data were analyzed by one-way ANOVA followed by the Bonferroni post-hoc test. Data were analyzed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA) with $P \leq .05$ as the cut-off for statistical significance between groups.

## 3. Results

### 3.1. Dimensions of the brain from rats fed with different milk

The weight and the length of the brains from rats fed with different types of milk did not show any difference (Supplementary Fig. S1).

### 3.2. DNA methylation changes in rat brain cortex induced by administration of different milk

To investigate whether different milk intake impacted brain DNA methylation profiles, we performed Reduced Representation Bisulfite Sequencing (RRBS) on brain cortex-derived DNA from rats supplied with different types of milk (human, cow, donkey) to their standard diet. We identified 2494 differentially methylated cytosines (DMCs) (FDR < 0.05, 1620 hypomethylated and 874 hypermethylated regions) induced by human milk intake (Fig. 1A), 2422 DMCs (FDR < 0.05, 1243 hypomethylated and 1179 hypermethylated regions) induced by donkey milk intake (Fig. 1B), 2616 DMCs (FDR < 0.05, 1654 hypomethylated and 962 hypermethylated regions) caused by cow milk intake (Fig. 1C). The annotation of these cytosines showed a similar distribution along genes, with 4% approximately located on promoter 1–3kb, 25% approximately in intron and a large fraction (67–69%) found in distal intergenic regions (Fig. 1D–F). The most significant differences are in the distribution of cytosines at 3′UTR, which is 0.64%, 1%, and 0.88% in human, donkey and cow milk-fed rats, respectively (Fig. 1D–F). RRBS data revealed that varied milk intakes produced different DMCs with a similar distribution in corresponding genome annotations, with almost 70% of the DMCs covering intergenic areas. Notably, rats fed donkey milk had higher methylation acquisition and lower methylation loss than rats fed cow or human milk (Fig. 1D–F). However, CpG hypermethylation is not always associated with

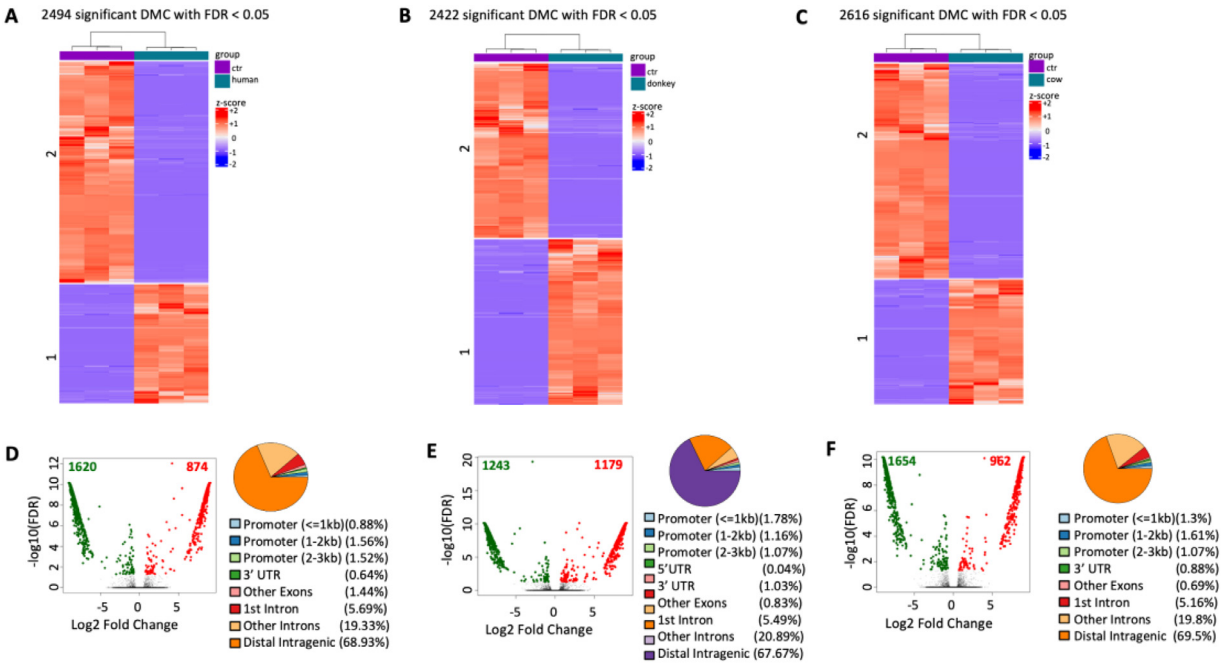



**Fig. 1.** Differentially methylated cytosines (DMCs) in human, donkey and cow milk-fed rats. (A–C) Heatmaps showing clustered DMCs from RRBS performed on human (A), donkey (B) and (C) cow (C) milk-fed rats compared to control group. (D–F) Volcano plot and distribution of hyper-methylated (increased methylation in red) and hypo-methylated (decreased methylation in green) DMCs across different genomic regions in (D) human, (E) donkey and (F) cow milk-fed rats. Data, significantly different from controls by Student unpaired t-test ($P < .05$), are from $n = 3$ animals/group.

silencing. The inhibition or activation of transcription by methylation is dependent on the gene segment analyzed. For example, hypermethylation at gene bodies is associated with gene expression [36], while hypermethylation of TSS or enhancers invariably leads to silencing [37]. Analysis of methylation along gene segments showed that rat fed cow milk had lower promoter methylation levels (40%) than animals fed human (43%) or donkey (44%) milk (Fig. S2A), with rat fed human milk having lower gene body methylation levels (47%) than animals fed donkey (56%) or cow (60%) milk (Fig. S2B). Thus, considering the nonrandom hypermethylated and hypomethylated imbalance on the genetic body, it is likely that different types of milk can modulate different sets of genes [38] also in the cerebral cortex.

### 3.3. Gene expression changes in rat brain cortex induced by administration of different milk

The results in Fig. 1 and S2 indicate that different kinds of milk induced different epigenetic changes, which may affect gene expression to turn genes on or off. To gain a better insight into the mechanisms linking milk intake and brain activity, we performed RNAseq on the brain cortex of the same animals. Approximately 70% of the 100 million reads we typically obtained per sample were uniquely mapped.

We identified 16 Differential Expressed Genes (DEGs) (FDR < 0.05, 9 downregulated and 7 upregulated genes) induced by human milk intake (Fig. 2A, D), 21 DEGs (FDR < 0.05, 7 downregulated and 14 upregulated genes) induced by donkey milk intake (Fig. 2B, E), 205 DEGs (FDR < 0.05, 161 downregulated and 44 upregulated genes) caused by cow milk intake (Fig. 2C, F) compared to control. Gene enrichment analysis on RNAseq data sets was performed to determine which molecular pathways were impacted. We discovered that human milk intake stimulated SRC-PRKCD-CDCP1 and BVR-PRKCD-ERK2 signaling that is associated with synaptic plasticity [39], neuronal differentiation [40] and oxidative-damage response [41] (Fig. 3A), while donkey and cow milk intake stimulated or repressed pathway associated with behavior, response to stimuli, nervous system development and synaptic signaling, respectively (Fig. 3B, C). It should be noted that rat fed human milk had lower levels of complement C4a and higher levels of Prkcd (protein kinase C, delta) genes, which drive synaptic pruning and short-term plasticity, respectively (Fig. 4D). Rat fed donkey milk had lower levels of CAST and higher levels of SynDIG1, which regulate Voltage-Gated $Ca^{2+}$ Channel Density and Excitatory Synaptogenesis, respectively (Fig. 3D). Cow milk fed rat had lower levels of Mdm4 and higher levels of H2aX genes, both of which are indicators of DNA damage (Fig. 3D).

Finally, we used a Smith-Ranking test to get insight into epigenetic alterations to correlate gene expression with methylation status that regulates gene expression (acquisition or loss of methylation). First, we chose genes using three criteria: (1) genes with a statistically significant *P*-value in DMCs, (2) genes with highly regulated RNAseq data, and (3) genes with a link between promoter methylation status and expression (the expressed genes will have demethylated promoters and vice versa). Supplementary Table 3 lists identified genes. Then, for RT-qPCR study validation, we identified five predicted RNAseq genes differently regulated relative to control. We have amplified *RAB18* and *CHRM4* genes in rats supplied with donkey milk vs control, *TIMM22* in rats supplied with human milk vs control, and *SLC17A6* and *CDHR*1 in rats treated with cow milk. Our data demonstrate that *RAB18* RNA levels decreased in rat brains treated with donkey milk compared to control, while *CHRM4, TIMM22, SLC17A6* and *CDHR*1 expression levels increased in rats supplied with the different milk (Fig. S3A–E). These data demonstrate a close correlation between epi-

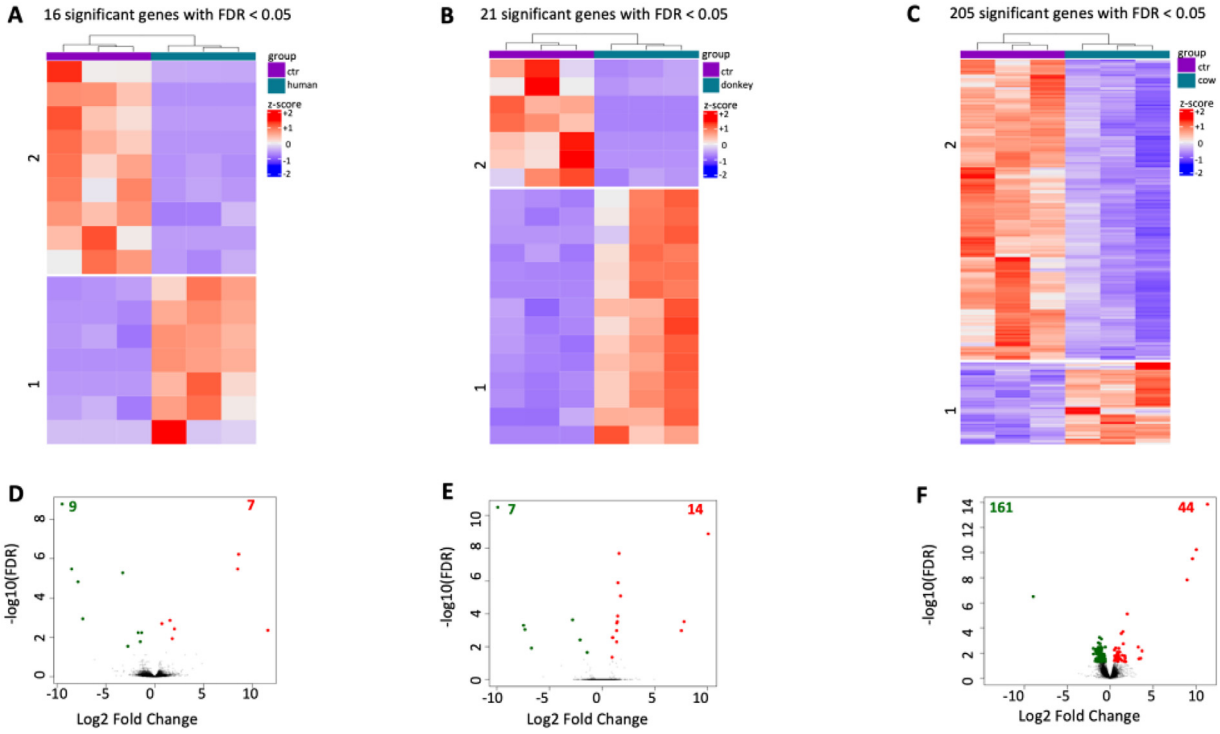
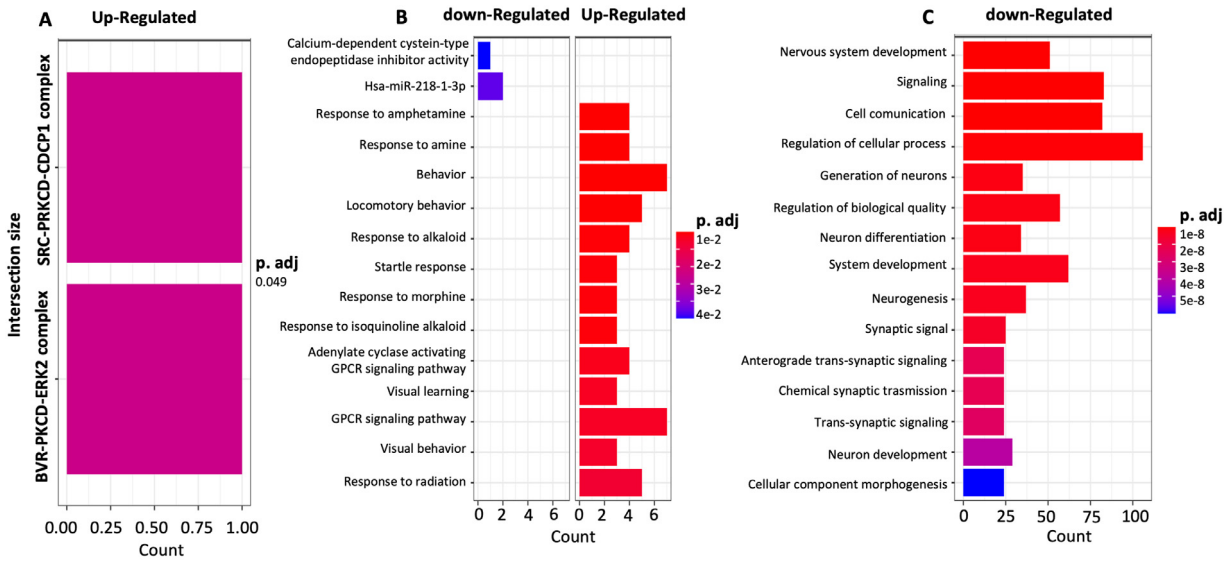



**Fig. 2.** Differentially expressed genes (DEs) in human, donkey and cow (C, F) milk-fed rats. (A–C) Heatmaps showing clustered DEGs of (A) human, (B) donkey and (C) cow milk-fed rats compared to control group. (D–F) Volcano plot of DEs showing repressed (decreased expression in green) or activated (increased expression in red) in human (D), donkey (E) and cow (F) milk-fed rats. Data, significantly different from controls by Student unpaired t-test ($P < .05$), are from $n = 3$ animals/group.




D

| Symbol | Log2foldChange | padj<= | Description | Group |
|---|---|---|---|---|
| Prkcd | 1.81 | 0.01 | protein kinase C, delta | human |
| C4a | -9.47 | 1.7e-09 | complement C4A | human |
| CAST | -6.71 | 0.01 | calpastatin | donkey |
| SynDIG1 | 1.38 | 0.001 | synapse differentiation inducing 1-like | donkey |
| Mdm4 | -1.24 | 0.03 | MDM4 regulator of p53 | cow |
| H2aX | 0.55 | 0.03 | H2A.X variant histone | cow |

**Fig. 3.** Enrichment analysis of the KEGG pathways. (A–C) show significant enrichment pathway stimulated by fed rats with human, donkey and cow milk. (D) reports the main genes modulated in the same animals.

genetic modification, gene expression and long-term milk intake consumption.

### *3.4. Mitochondrial functions, efficiency and oxidative stress in rat brain cortex following administration of different milk*

Gene ontology transcription analysis indicates that the administration of different milk affects pathways related to oxidative damage response. Since these pathways are tightly linked to mitochondria functions, we investigated mitochondrial respiration in the brain cortex.

We measured the mitochondrial respiration in the brain cortex in the presence of succinate/rotenone or palmitoyl-carnitine/malate as substrates. In the presence of succinate, state 3 mitochondrial oxygen consumption was significantly increased in the HM and DM groups when compared to the control and CM-treated groups. CM showed the lowest oxygen consumption rate (Fig. 4A). When palmitoyl-carnitine was used as a substrate, state 3 respiration was significantly increased in mitochondria isolated from the CM-treated animals compared to the other groups. No differences we observed in state 4 respiration among all groups, both in the presence of succinate and palmitoyl-carnitine (Fig. 4B). The respiratory control ratio (RCR) values indicated high-quality mitochondrial preparations (data not shown). The CPT activity, a mitochondrial system that enables the transfer of fatty acids from the cytoplasm to the mitochondrial matrix for beta-oxidation, significantly increased in the DM and HM-treated animals compared to controls and CM-treated rats (Fig. 4C). No variation between the groups was found in state 4 respiration in the presence of oligomycin and maximal FCCP-stimulated respiration (data not shown). Consequently, the mitochondrial energy efficiency in the brain cortex, assessed as the degree of coupling, was similar in all groups of animals (Fig. 4D). The HM-treated rats exhibited a decreased $H_2O_2$ yield compared to other groups (Fig. 4E). In parallel, we observed a significant increase in SOD activity in DM and HM rats compared to CM and control groups (Fig. 4F). In addition, dietary supplementation with DM or HM reduced oxidative stress and improved the antioxidant state in the brain cortex homogenate. Indeed, the MDA and ROS contents were significantly lower in the DM- or HM-treated rats than in the CM-treated and control rats (Fig. 4G, H). MDA is a highly reactive compound that results from lipid peroxidation of polyunsaturated fatty acids, commonly used as a marker of oxidative stress and tissue damage [42]. CM-treated animals showed higher MDA and ROS values than other groups (Fig. 4G, H). In addition, the GSH levels significantly increased in DM or HM group, compared to CM and control animals. In contrast, no differences we observed in GSSG content among groups (data not shown), resulting in an increased GSH/GSSG ratio in the HM and DM groups (Fig. 4I). GSH/GSSG ratio is an indicator of cellular health and can be used as a marker for oxidative stress. In CM-treated rats, we observed the lowest GSH content and GSH/GSSG ratio (Fig. 4I).

### *3.5. Modulation of synaptic proteins in the brain cortex of rats fed with different milk*

Gene ontology transcription analysis also indicates modulation of synaptic organization and transmission pathways following dif-

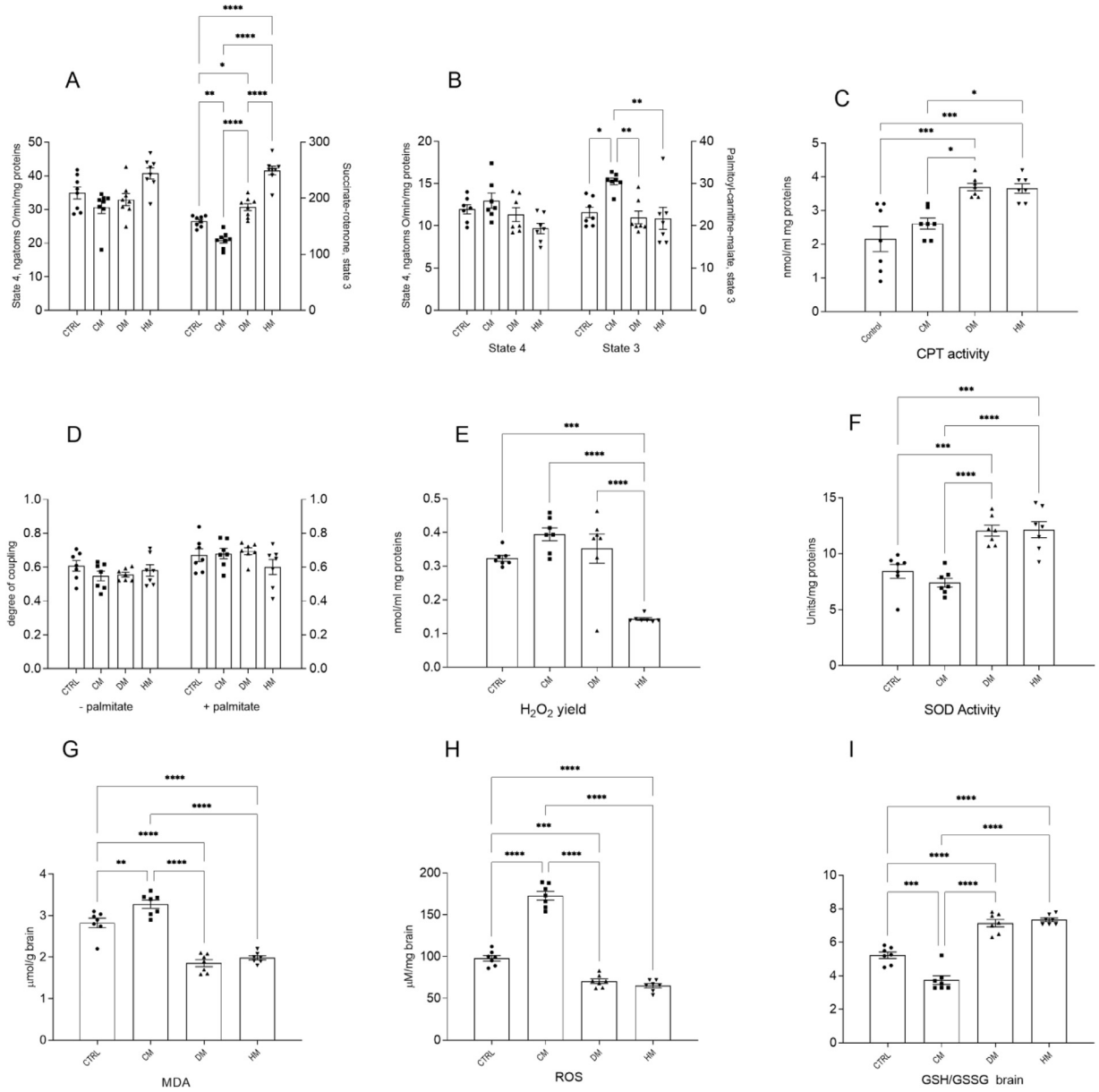



**Fig. 4.** Effects of oral administration of human, donkey, and cow milk on respiratory parameters, energy efficiency and oxidative stress in mitochondria isolated from brain cortex. The graphs show the mitochondrial respiration rates measured in the presence of succinate (A) or palmitoyl-carnitine (B) as substrates, the carnitine-palmitoyl transferase (CPT) activity (C), the degree of coupling in presence or absence of palmitate (D), the $H_2O_2$ yield (E), the superoxide dismutase (SOD) activity (F), the MDA levels (G), the ROS levels (H), the GSH/GSSG ratio (I). Data are presented as means ± SEM from $n = 7$ animals/group. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$ indicate statistically significant differences calculated by one-way Anova followed by Bonferroni post-hoc test among the groups.

ferent milk administrations. Thus, by western blot, we evaluated the expression of synaptic proteins in the brain cortex of rats fed with different types of milk. In particular, we examined two proteins playing a key role in synaptic transmission: synaptophysin, a transmembrane protein of the synaptic vesicle, and syntaxin, a protein of the presynaptic membrane involved in exocytosis [43] (Fig. 5). The level of synaptophysin significantly increases in HM groups compared to CM, while in DM is higher than in CM and lower than in HM, but the differences are not significant (Fig. 5A, B). The level of syntaxin in HM groups is significantly higher than in the other two groups (Fig. 5A, C). Altogether, these results indicate a positive impact of HM supplementation at the synaptic level.

### *3.6. Mitochondrial functions, efficiency and oxidative stress in the synaptosomal fraction from the brain of rats fed with different milk*

Based on gene ontology and western blot results showing a strong influence of milk administration at the synaptic level and considering the crucial role played by synaptic mitochondria in supporting synaptic functions and plasticity, we examined the mitochondrial function of the synaptosomal fraction from the brain cortex using the Seahorse XFp (Seahorse Bioscience, North Billerica, MA, USA). It was performed in real-time at the basal level and following a sequential addition of mitochondrial respiration modulators: oligomycin, FCCP, and a combination of antimycin A and rotenone. The Cell Mito Stress Test results on synaptosomal frac-

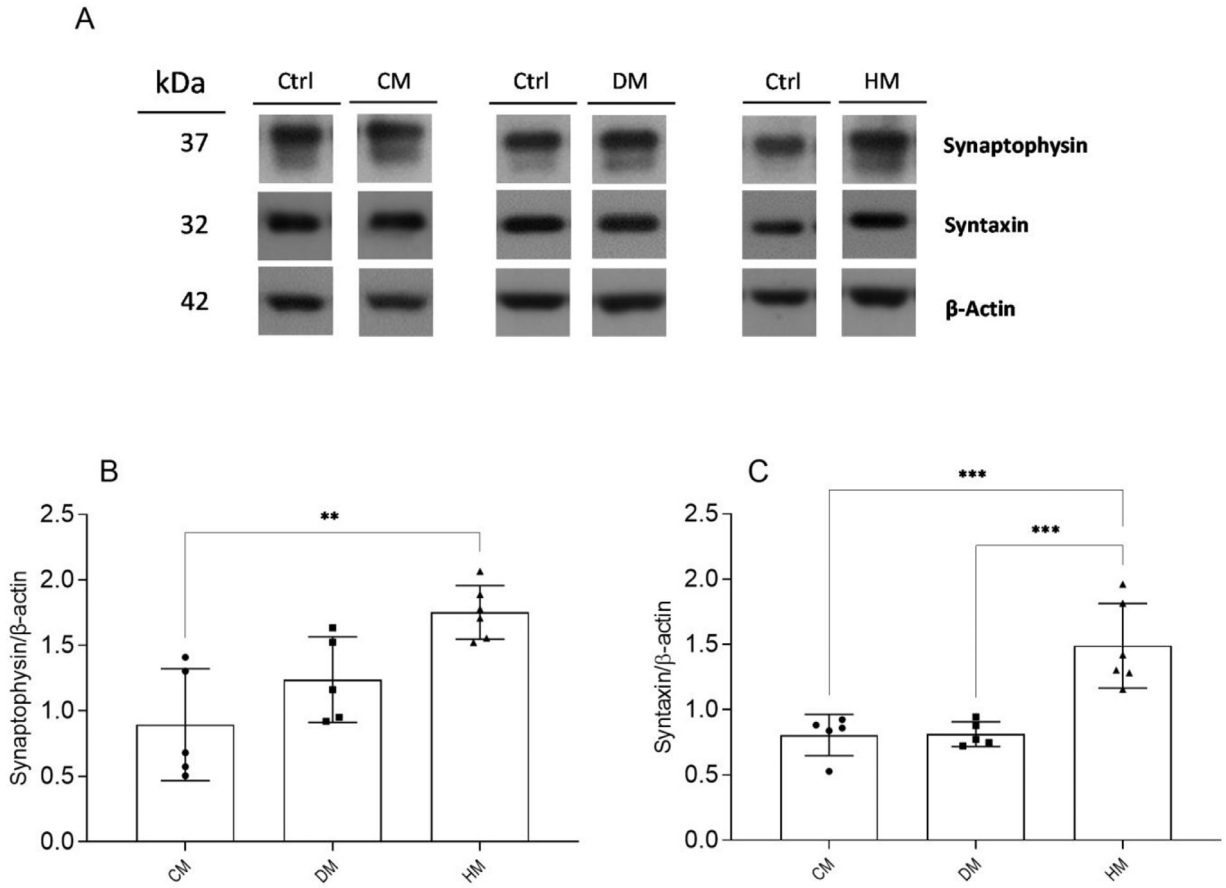



**Fig. 5.** Expression levels of synaptic proteins in the brain cortex of rat fed with different type of milk. (A) Representative image of western blot analysis using synaptophysin and syntaxin antibodies. Expression levels of synaptophysin (B) and syntaxin (C) normalized on $\beta$-actin. Data are presented as ratio between group value/controls value (means ± SEM, $n = 4/5$ animals/group). $^{**}P < .01$; $^{***}P < .001$ indicate statistically significant differences calculated by one-way Anova followed by Bonferroni post-hoc test among the groups. Ctrl, control diet; CM, cow milk; DM, donkey milk; HM, human milk.

tion showed a significant and progressive increase in basal and maximal respiration in DM and HM rats compared to controls. The CM group exhibited the lowest basal and maximal respiration values than other animal groups (Fig. 6A, B). These results are consistent with the increased ATP production in DM and HM animals compared to the control and CM rats (Fig. 6C). Mitochondrial proton leakage significantly increased in DM and HM rats compared to control or CM rats, and consequently, the coupling efficiency diminished. In contrast, CM showed the lowest value of proton leak and, consequently, a higher value of coupling efficiency than other groups (Fig. 6D, E). In the synaptosomal fraction, the oxidative stress and the antioxidant state were similar to those observed in the brain cortex homogenate. Indeed, the MDA and ROS contents were significantly lower in the DM- or HM-treated rats compared to CM-treated and control animals, while the highest values were observed in CM-treated rats (Fig. 6F, G). Also, we observed that the levels of GSH significantly increased in DM and HM compared to other groups. At the same time, no differences were found in GSSG content among differently treated groups (data not shown), resulting in an increased GSH/GSSG ratio in the HM and DM groups (Fig. 6H).

### *3.7. Inflammatory state in brain cortex and synaptosomal fraction from rats fed with different milk*

The reduced (GSH)-to-oxidized (GSSG) glutathione ratio represents a dynamic balance between oxidants and antioxidants. In fact, GSH protects cells against exogenous and endogenous harmful molecules including reactive oxygen and nitrogen species (ROS/RNS), limiting the damaging effects of oxidative/nitrosative stress [44]. Moreover, intracellular GSH depletion represents the first event of the inflammatory signaling process [45]. Interestingly, we observed similar modulation of inflammatory state in the brain cortex (Fig. 7, panel A) and synaptosomal fraction (Fig. 7, panel B) from rats fed with different milk. Indeed, TNF-$\alpha$, IL-1$\beta$, and IL-6 levels were significantly lower in DM- and HM-treated rats compared to CM-treated and control rats. In CM-treated rats, we observed the highest levels of inflammatory markers (TNF-$\alpha$, IL-1$\beta$, IL-6). In addition, the IL-10 levels, an anti-inflammatory cytokine, were significantly increased in DM- and HM-treated rats and significantly decreased in CM-treated animals (Fig. 7).

## 4. Discussion

In this study we investigated, for the first time, epigenetic modulation, at the brain levels, following administration to rats of milk from different animal species. We showed that changes in animal feeding affect DNA methylation in rat brain cortex leading to modulation of gene expression and brain mitochondrial metabolism [46]. The intake of different types of milk induced differences in promoter methylation (cow 40%, human 43%, and donkey 44%) and gene body methylation levels (cow 47%, human 60%, and donkey 56%), which resulted in a different gene expression. In particular, feeding with both human and donkey milk may act on pathways

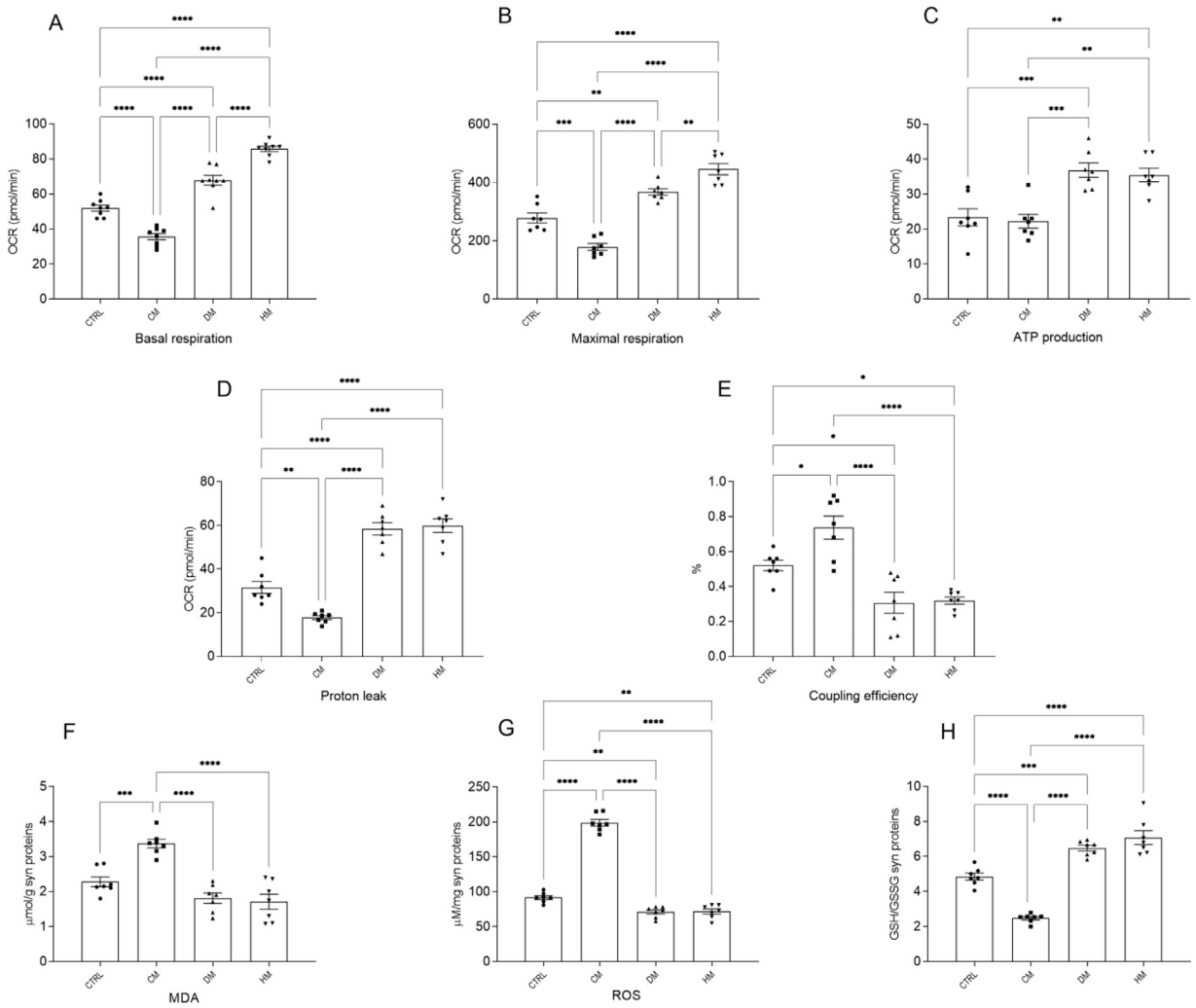



**Fig. 6.** Effects of oral administration of human, donkey and cow milk on mitochondrial respiration parameters and oxidative stress in synaptosomal fraction. The graphs show the basal respiration (A), the maximal respiration (B), the ATP production (C), the proton leak (D) and the coupling efficiency (E). The MDA (F), the ROS levels (G) and the GSH/GSSG ratio (H) in the synaptosomal fraction are also showed. Data are presented as means ± SEM from $n = 7$ animals/group. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$ indicate statistically significant differences calculated by one-way Anova followed by Bonferroni post-hoc test among the groups.

stimulating synapse formation and neuronal transmission: HM decreases the expression level of complement C4a factor that controls synaptic pruning, while DM modulates the expression level of CAST and SynDIG1 that have been reported to play a critical role in synapse maturation recruiting AMPA receptors to nascent synapses [47]. Interestingly, we observed the highest levels of gene modulation in the brain cortex of CM rats. However, among the modulated genes, the reduction of Mdm4 levels and the increase of the H2aX levels were observed, which are both DNA damage markers. These results suggest that the consumption of human milk positively affects synaptic plasticity through epigenetic mechanisms, including chromatin remodeling and DNA methylation. One candidate molecule to mediate the beneficial effects of human milk administration in synaptic area is myo-inositol, which has been demonstrated to be present at higher level in HM and DM compared to CM [48,49]. Indeed, myo-inositol, acting in a dose-dependent manner, promote neuronal connectivity in human excitatory neurons as well as cultured rat neurons [50]. Our findings, indicating the epigenetic role of milk intake, are in line with the Melnik's group work [51], proposing that milk may function as an "epigenetic transfection mechanism" by delivering RNA-containing exosomes [52] that regulate the infant's epigenome [53,54].

Brain plasticity requires a great energy supply, tightly depending on mitochondria activity, especially at synaptic levels [55]. Our findings demonstrated that different kinds of milk affect in different ways brain cortex bioenergetic. In particular, human milk and donkey milk has beneficial effects, improving mitochondrial function and efficiency, reducing inflammation and oxidative stress through epigenetic modifications, including DNA methylation. Conversely, cow milk administration induces an increase in inflammatory state and oxidative stress in brain cortex, associated with alteration of mitochondrial functions. Interestingly, in brain cortex, we observed that human milk increases the expression levels of two presynaptic proteins, synaptophysin and syntaxin, suggesting an effect of milk administration in synaptic areas. Therefore, we investigated the mitochondrial functions and oxidative stress in synaptosomes, and we detected a beneficial effect of human and donkey milk. Notably, the dysfunctions of presynaptic mitochondria may be one of the mechanisms contributing to cognitive decline in neurodegenerative diseases [56].

Donkey and human milk administration improves brain mitochondrial oxidation as demonstrated by a higher state 3 oxygen consumption rate when succinate was used as substrate. On the contrary, CM rats showed the lowest mitochondrial oxidative ca-

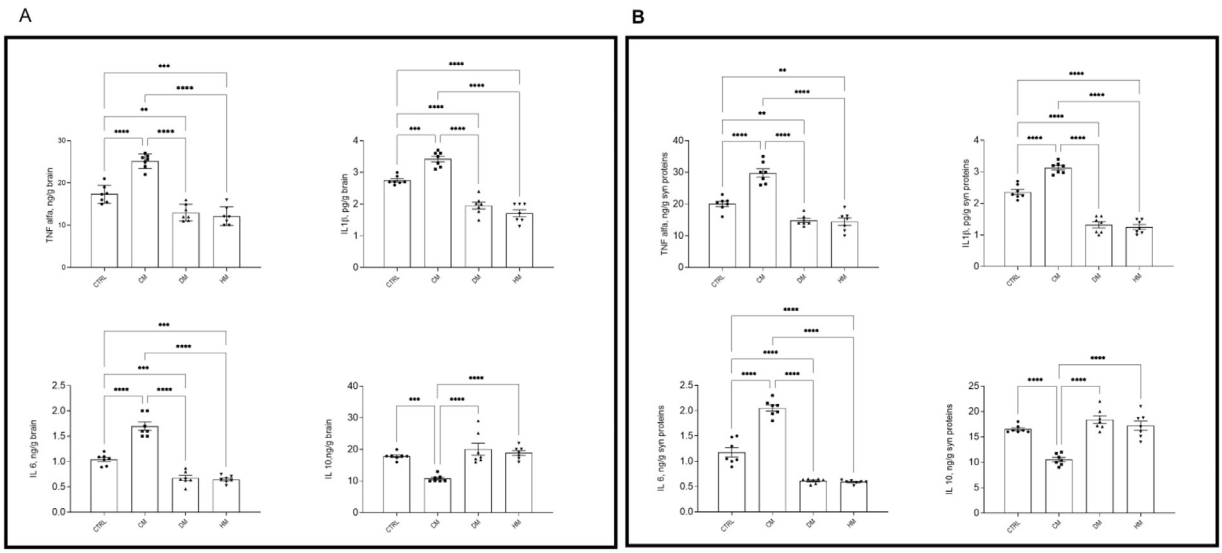



**Fig. 7.** Effects of oral administration of human, donkey and cow milk on pro-inflammatory parameters in cerebral cortex tissue and in synaptosomal fraction. (A) TNF$\alpha$, IL-1$\beta$, IL-6 and IL-10 levels measured in the cerebral cortex. (B) TNF$\alpha$, IL-1$\beta$, IL-6 and IL-10 levels measured in the synaptosomal fraction. Data are presented as means ± SEM from $n = 7$ animals/group. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$ indicate statistically significant differences calculated by one-way Anova followed by Bonferroni post-hoc test among the groups.

pacity. Nonetheless, in the presence of palmitoyl carnitine, assessing the oxidation capacity of fatty acids, the highest respiratory rate was detected in CM group, although this rise does not correlate with an increase in CPT activity. This increased lipid oxidation may not be sufficient to handle an excess of FFAs, resulting in triglyceride storage in adipose tissue, and in ectopic tissue such as skeletal muscle and liver [7,16]. The high level of lipid oxidation is generally associated with increased oxidative stress. Indeed, the CM group showed increased mitochondrial oxidative stress parameters (hydrogen peroxide yield, aconitase activity, and GSH/GSSG ratio). This effect can be due to an increased fatty acid oxidation rate, resulting in NADH and FADH2 production, electron delivery to the respiratory chain, and concomitant respiratory chain impairment (indicated by the decrease in succinate State 3 oxygen consumption). As a result, the electron flow is partially blocked, leading to an increase in ROS production. The improved brain mitochondrial function and the low oxidative stress observed in DM- and HM-treated animals mirrors what has already been verified in other organs [6,7,16]. This impact on metabolic processes could be ascribed to the peculiarities of the composition of different types of milk [5]. HM and DM have high concentration of conjugated linoleic acids (CLA), $\omega$-3 polyunsaturated fatty acids and butyrate, which are able to modulate the mitochondrial function and antioxidant/detoxifying pathways [7,57,58]. Moreover, high concentrations of palmitic acid in the sn-2 position of triacylglycerol backbone in HM and DM [59] increase tissue levels of the N-oleoylethanolamine (OEA). OEA acts as a gut-to-brain signal to control food intake and metabolism [60], and it is an endogenous ligand of the nuclear receptor PPAR-$\alpha$. The activation of this receptor increases peroxisomal and mitochondrial oxidation and leads to anti-inflammatory and antioxidant effects [61,62].

This modulation of bioenergetic profile by different milk administration was also confirmed in the synaptosomal fraction of the same group of animals. Since synaptic mitochondria are crucial for supplying energy to support synaptic functions and plasticity, defects in these organelles may result in synaptic failure, a typical feature of neurodegenerative diseases. Our results highlighted that the CM group exhibited the lowest basal and maximal respiration values. In contrast, a significant increase was observed in DM and HM rats, consistent with an increased ATP production. In particular, significant differences in synaptic mitochondrial proton leakage were observed in different experimental groups: CM showed a strong decrease in proton leak and a consequent higher coupling efficiency compared to all other groups of animals, while DM and HM rats showed the highest proton leak value and a consequent lowest coupling efficiency. As proton leak depicts the protons that migrate into the matrix without producing ATP, the coupling of substrate oxygen and ATP generation is incomplete. Previously, it has been demonstrated that mitochondrial uncoupling is protective by mitigating cell ROS production [63]. Accordingly, we observed a decreased oxidative stress in synaptosomes from DM and HM groups. It is noteworthy that mitochondria are identified as “sentinel” organelles, capable of detecting cellular insults and orchestrating inflammatory responses [64]. In particular, mitochondrial ROS may be the factor enabling mitochondria to switch from low-grade inflammation to prolonged inflammatory responses, depending on the intensity of the insult [65]. Interestingly, the administration of DM and HM reduces the levels of key proinflammatory markers and increases the level of anti-inflammatory markers in both the cerebral cortex and the synaptic fraction.

Altogether, our data indicated that administering milk from different species to rats selectively modulates brain mitochondrial functions, ROS production and synaptic function, suggesting a possible link with pathological events of the nervous system. Interestingly, it has been reported a possible link between persistent cow milk consumption and dysfunctions related to an increased risk of Parkinson’s disease (PD) [66]. PD is a neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra (SN) of the midbrain leading to decreased striatal levels of dopamine and impaired motor control. Some of PD hallmarks are mitochondrial dysfunction, oxidative stress, and abnormal aggregation of $\alpha$-synuclein, a neuronal protein mainly localized to presynaptic terminals, which is involved in vesicles budding and recycling [67–70]. Increased evidence underlines that hypomethylation of the promoter of $\alpha$-synuclein gene increases the expression of the protein, affecting the PD pathogenesis [71,72]. Interestingly, it has been demonstrated that one of miRNAs most abundantly expressed in the bovine milk exosomes is miRNA-148a that down-

regulates DNA methyltransferase 1, promoting DNA hypomethylation and consequently increasing gene expression [73]. Thus, it is possible to hypothesize that the $\alpha$-syn overexpression characterizing PD, depends on persistent promoter demethylation induced by the consumption of pasteurized cow milk via transfer of exosomal microRNA-148a-3p [66].

Another important aspect to consider is the potential influence of gut microbiota in mediating the brain effects of milk administration. Indeed, we previously showed that changes in the gut microbiota can be responsible for the improvements in body composition, metabolic homeostasis and inflammatory state due to administration of human and donkey milk to rats. In particular, it was demonstrated that the supplement of these two types of milk resulted in the enrichment, in the gut microbiota, of Bacteroides and Parabacteroides, two genera that are associated to anti-inflammatory effects [16]. Accordingly, we previously demonstrated increased concentrations of fecal butyrate in HM- and DM-treated rats [16]. Thus, it is possible to hypothesize that the beneficial effects of HM and DM may depend on the butyrate ability to modulate the mitochondrial function and Nrf2–ARE pathway [74,75]. However, further studies are necessary to precisely identify the molecules responsible for these beneficial effects. More emphasis should also be placed on comparing the effects of breastfeeding *vs* artificial infant formula feeding based on milk from different sources. Previous studies demonstrated that breastfeeding showed modulatory effects on the expression level of obesity-predisposing genes (FTO, CPT1A, and PPAR-$\alpha$) in peripheral blood mononuclear cells (PBMCs) [76]. In this context, it is relevant to consider the correlation between milk consumption and species-specific activation of mTORC1 signaling, a central hub controlling cell signaling, growth and proliferation [77]. Another important factor is the milk content of branched chain aminoacids and glutamine, critical components increasing FTO expression [78]. Moreover, milk is known as a postnatal nutritional factor that mediates immune-stimulatory functions [79], with the bioactive exosomal miRNAs regulating long-term stable expression of FoxP3, the key transcription factor of Tregs [80]. These results emphasize the complex pathways activated by milk administration through epigenetic mechanisms, which deserve more extensive investigations.

## 5. Conclusion

Nowadays, it is well-accepted that dietary regimen affects brain health, and the appropriate diet can ameliorate some symptoms of neuropathologies [81]. Although no single nutritional component has been identified as crucial in improving brain wellness, eating habits and the synergistic interaction between different nutrients seem beneficial in preventing brain disorders [82]. For these reasons, it is conceivable that consuming foods rich in bioactive constituents, essential in the most critical phases of life (childhood and aging), such as milk, can influence the brain's bioenergetic mechanisms. Thus, our results, indicating the positive effects of human and donkey milk on brain mitochondrial functions through epigenetic regulation, open a new perspective in dietary approach for prevention or even for treatment of neuropathologies.

## Declaration of generative AI

Not applicable.

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## Declaration of competing interest

The authors declare that there are no conflicts of interest.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2024.109624.

## CRediT authorship contribution statement

**Antonia Feola:** Writing – original draft, Methodology, Investigation, Conceptualization. **Gina Cavaliere:** Methodology, Data curation. **Fabiano Cimmino:** Methodology, Data curation. **Angela Catapano:** Methodology. **Eduardo Penna:** Methodology. **Giovanni Scala:** Visualization, Software, Data curation. **Luigi Greco:** Supervision, Investigation. **Luca Bernardo:** Resources. **Antonio Porcellini:** Supervision, Conceptualization. **Marianna Crispino:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Conceptualization. **Antonio Pezone:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Investigation. **Maria Pina Mollica:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

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