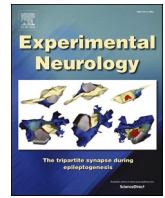




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Research paper

Ketogenic diet ameliorates autism spectrum disorders-like behaviors via reduced inflammatory factors and microbiota remodeling in BTBR T⁺ Itpr3^{tf}/J mice

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ABSTRACT

Autism Spectrum Disorder (ASD) is increasing, but its complete etiology is still lacking. Recently, application of ketogenic diet (KD) has shown to reduce abnormal behaviors while improving psychological/sociological status in neurodegenerative diseases. However, KD role on ASD and underlying mechanism remains unknown. In this work, KD administered to BTBR T⁺ Itpr3^{tf}/J (BTBR) and C57BL/6J (C57) mice reduced social deficits ($p = 0.002$), repetitive behaviors ($p < 0.001$) and memory impairments ($p = 0.001$) in BTBR. Behavioral effects were related to reduced expression levels of tumor necrosis factor alpha, interleukin-1 β , and interleukin-6 in the plasma ($p = 0.007$; $p < 0.001$ and $p = 0.023$, respectively), prefrontal cortex ($p = 0.006$; $p = 0.04$ and $p = 0.03$) and hippocampus ($p = 0.02$; $p = 0.09$ and $p = 0.03$). Moreover, KD accounted for reduced oxidative stress by changing lipid peroxidation levels and superoxide dismutase activity in BTBR brain areas. Interestingly, KD increased relative abundances of putatively beneficial microbiota (*Akkermansia* and *Blautia*) in BTBR and C57 mice while reversing the increase of *Lactobacillus* in BTBR feces. Overall, our findings suggest that KD has a multifunctional role since it improved inflammatory plus oxidative stress levels together with remodeling gut-brain axis. Hence, KD may turn out to be a valuable therapeutic approach for ameliorating ASD-like conditions even though more evidence is required to evaluate its effectiveness especially on a long term.

Abbreviations: ASD, autism spectrum disorder; CD, controlled diet; DI, discrimination index; HIP, hippocampus; IL, interleukin; KD, ketogenic diet; LDT, light dark box test; NOR, novel object recognition test; PCoA, Principal Coordinate Analysis; PFC, prefrontal cortex; ROS, reactive oxygen species; SI, sociability index; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TNF α , tumor necrosis factor alpha..

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1. Introduction

Autism Spectrum Disorder (ASD) is one of the most complex neurodevelopmental syndromes having an enormous impact during the childhood stage in terms of prevalence, morbidity, family, and society burdens (Lyal et al., 2017). The main features characterizing such a disorder include impaired social interaction and communication ability, coupled with restricted and repetitive patterns of behaviors, which constitute the behavioral basis of its diagnosis (Kodak and Bergmann, 2020). In addition, psychiatric and medical comorbidities include gastrointestinal symptoms, anxiety, plus cognitive impairments that seem to occur at different frequencies thus overlapping and masking the main features of ASD (Alò et al., 2021; Shu et al., 2022). At date, both pathological processes and molecular etiology of this disorder have not yet been fully established due to genetic events interacting negatively with environmental factors (Cheroni et al., 2020). Consequently, therapeutic approaches used for their treatment are often limited and do not act on main symptoms of this neurodevelopmental disorder. From several studies, it appears that the different inflammatory events exert a key role on the pathogenesis of ASD and above all on the majority of its behavioral symptoms (Lee et al., 2016; Abruzzo et al., 2019; Tzanoulidou et al., 2022). Indeed, ASD patients largely display altered inflammatory states and immune abnormalities, showing very high levels of pro-inflammatory cytokines, such as interleukins (IL) and the tumor necrosis factor alpha (TNF- α), together with microglia activation (Ahmad et al., 2019). It is also known that under physiological conditions, microglia regulate the formation, pruning and elimination of synapses, together with their plasticity through an appropriate release of cytokines and chemokines. At the same time, an abnormal activation of microglia has been shown to induce an altered release of these factors and reactive oxygen species (ROS) leading to loss of neuronal functions and altered synaptic density (Simpson and Oliver, 2020). In particular, notable oxidative stressful conditions have been observed throughout the pathogenesis of ASD (Pangrazzi et al., 2020), which leads to an excessive ROS production with neuro-inflammatory dysfunctions accounting for the abnormal behavioral performances (Teleanu et al., 2022).

Increasing evidence points to the intestinal microbiota and the central nervous system (CNS) (i.e. gut microbiome–brain axis) as a principal target for the altered behavioral events in ASD (Avolio et al., 2022). It seems that microbiota can trigger inflammatory process throughout the release of metabolites and pro-inflammatory mediators, which reach CNS through the bloodstream thus inducing neuro-inflammatory events. The gut microbiota has emerged as an important factor in the brain development, capable of controlling the various neurodevelopmental brain activities, as suggested by its ability to induce the synthesis and secretion of neuroactive factors like serotonin, dopamine, GABA and glutamate, which are able to interfere with neuronal pathways in the different brain regions as well as with neuronal survival and trafficking events (Di Vito et al., 2014; Chen et al., 2021).

It is already known that the individual's metabolic state can strongly influence neuronal activities that in turn modify neurophysiological conditions of the brain and consequently behavioral performances. In this context, a ketogenic diet (KD) by drastically reducing carbohydrates, while increasing proteins and especially fats, forces body to use them as a source of energy so leading to diminished inflammatory events of numerous neurodegenerative syndromes (Jiang et al., 2022). Accumulating evidence have also shown that KD is able to exert a valuable role for the treatment of multiple diseases including epilepsy, depression, migraine, Alzheimer's and Parkinson's diseases (Pietrzak et al., 2022). Furthermore, KD has been correlated to improved behaviors in ASD (Ruskin et al., 2017) very likely via a modulation of gut microbiota conditions thus contributing to maintain homeostasis within CNS (Kaviyaran et al., 2022).

On the basis of the above indications, it was the aim of this study to investigate KD effects on typical ASD behaviors (social and cognitive

deficits, repetitive behaviors and anxiety) in BTBR T⁺ Itpr3^{fl}/J (BTBR) mice, a valuable mouse model of idiopathic ASD (Arakawa, 2021). Specifically, behavioral results were correlated to plasma and brain expression of TNF- α , IL-1 β and IL-6, which are main mediators of inflammation and are implicated in the bidirectional pathway between gut and brain (Zhao et al., 2021; Avolio et al., 2022). In addition, oxidative stress levels (Thiobarbituric Acid Reactive Substances, TBARS; superoxide dismutase, SOD) in the prefrontal cortex (PFC) plus hippocampus (HIP) as well as gut microbiota changes were also detected. The results of these evaluations are aimed to establish possible pathways plus specific molecular factors by which KD ameliorates neurological-related disturbances in BTBR mice thus further supporting its possible role as an appropriate therapeutic approach not only for the treatment of the above neurodegenerative diseases but also for ASD.

2. Materials and methods

2.1. Animals and treatments

Male offspring of BTBR breeding pairs and the social counterparts C57BL/6J (C57) mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed and bred in plastic cages (28 cm wide \times 17 cm long \times 12 cm high) in a humidity (60%) and temperature-controlled (22 \pm 2 $^{\circ}$ C) room with a 12-h light/dark cycle (lights on at 07:30 am) plus free access to food and water. The offspring were kept with the dam until weaning at postnatal day 22 \pm 1. After weaning, mice of both strains were grouped 3–5 per cage and assigned randomly to a controlled diet (CD, 2019 Teklad Global Diet - 9% fat, 19% protein, 44.9% carbohydrate; Envigo RMS, Udine, Italy) or to a KD (PF4390 diet, 67.70% fat, 15.90% protein, 1% carbohydrate; Mucedola, Milan, Italy) for five weeks, an intervention period similar to that reported by others (Qin et al., 2022). Animal maintenance and experimental procedures were carried out in compliance with ethical provisions for care and use of Laboratory Animals reported in the legislative law n $^{\circ}$ 26 (04-03-2014) and authorized by the National Committee of the Italian Ministry of Health. All measures were taken to minimize animal suffering and reduce the number of experiments.

2.2. Behavioral analysis

At the end of 4 weeks of treatment, 8 mice for each group were assessed for the different behavioral tests that lasted for 1 week until the end of KD treatment. Testing was conducted between 10 am and 5 pm in an appropriate behavioral testing room in which mice were acclimatized for 60 min prior to each test. The following tests were sequentially performed: Light Dark Test (LTD), Three-Chamber Sociability Test, Self-Grooming Test, and Novel Object Recognition (NOR) test. One test was handled per day until all assessments were completed. Ethanol was used to clean the testing apparatus after each trial.

Behavioral data were analyzed using EthoLog (version 2.2.5, Visual Basic, São Paulo, Brasil), a specific software that was linked via an overhead camera (SONY, DSC-W310) and tracked the movement of each subject during all observation sessions as previously reported (Zizza et al., 2014; Zizza et al., 2022).

2.2.1. Light dark test

All mice groups were exposed to LTD apparatus for evaluating anxiety-like behaviors as previously described (Avolio et al., 2019; Alò et al., 2022). LTD consisted of a box with two compartments, which were interconnected by an opening: a first arena composed of a small and dark plastic compartment (16 \times 16 \times 16 cm) while a second arena containing a large translucent and white illuminated compartment (25 \times 25 \times 30 cm). Each mouse was placed in the illuminated compartment, facing away from the opening, and allowed to explore the box for 5 min. Dependent variables included time spent in the light area, the first latency to cross to the dark area (all four paws in chamber) and the total

number of transitions between compartments were used as indexes of anxiety.

2.2.2. Three-chamber sociability test

To verify the effect of KD on sociality events, a Three-Chamber Sociability Test was handled as previously described (Zizza et al., 2022) with some modifications. The test was performed in a rectangular apparatus (23 × 40 × 22 cm) divided into 3 interconnected chambers. The task included two 10 min exploration trials, each starting from the middle chamber. For this part, the following steps were carried out: 1) Habituation trial: animals were allowed to freely explore all three empty chambers. 2) Sociability trial: a stranger mouse (same age and no previous contact with the subject) was put underneath a wire cage, randomly placed in the left or right compartment, while another identical empty cage, containing a novel object, was placed on the other side. This approach allowed us to distinguish the different sociability parameters by measuring the amount of time spent in each chamber as well as time exploring the novel object or the stranger mouse. Exploration behavior was defined as sniffing or touching either wire cages at <2 cm from snout. Sociability Index (SI) was calculated for intergroup comparisons according to the following formula:

$$SI = \frac{\text{time exploring stranger} - \text{time exploring novel object}}{\text{time exploring stranger} + \text{time exploring novel object}}$$

2.2.3. Self-grooming test

The Self-Grooming test was handled to assess repetitive behavioral activities (McFarlane et al., 2008). Mice were placed individually in a clean, empty plastic cage (33 × 15 × 13 cm) without bedding. After a 10 min habituation period, the cumulative time spent grooming all body parts was registered with a stopwatch during an interval of 10 min.

2.2.4. Novel object recognition test

NOR test was performed to assess animal ability to discriminate between a familiar and novel object according to previous works (Alò et al., 2021; Fazzari et al., 2018) plus modifications. Mice were individually habituated in an empty polycarbonate cage (50 × 50 × 30 cm) to freely explore the environmental layout of the cage for 10 min. The next day, the mouse was introduced into the arena with its head positioned opposite to two identical cylindrical blocks (indicated as familiar objects = 8 cm high × 2 cm diameter) and allowed to explore them for 5 min. After one hour, one of the familiar objects was exchanged with a novel object (a rectangular block = 8 × 2.5 × 2 cm) and then the mouse was reintroduced into the arena for 5 min. The sessions were video-recorded, and time spent exploring the objects was scored. Exploration behavior was defined as sniffing or touching either object at <2 cm from snout, while sitting on the object was not considered exploration. Recognition ability was evaluated as a Discrimination Index (DI), using the following formula:

$$DI = \frac{\text{time exploring novel object} - \text{time exploring familiar object}}{\text{time exploring novel object} + \text{time exploring familiar object}} \quad (\times 100)$$

2.3. Pro-inflammatory cytokine evaluation

IL-1 β , IL-6 and TNF α proteins were determined in the brain and plasma samples via application of Enzyme-Linked Immunosorbent Assay (ELISA) as previously described (Godbout et al., 2005). In brief, mice ($n = 6/\text{group}$) were sacrificed, and blood was collected by cardiac puncture. Blood was centrifuged at 12500 rpm (4 °C) for 15 min at 4 °C, then plasma was collected in heparinized tubes and frozen (-20 °C) until further analyzes. Brains were removed and PFC plus HIP were dissected out, then homogenized in lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, plus 1% nonidet P-40), centrifuged (12,500 rpm at 4 °C for 30 min) and clarified lysates were assayed for total protein content (Bio-Rad, Cambridge MA).

Plasma samples and homogenates of brain regions for each experimental group were used to estimate the concentration of IL-1 β , IL-6 and TNF- α cytokines. Cytokines were determined by using a “Mouse TNF alpha ELISA Kit” (Cat. # MBS825075; MyBioSource, San Diego, USA), a “Mouse IL-1 β ELISA kit” (RAB0275) and a “Mouse IL-6 ELISA kit” (RAB0309; Sigma Aldrich, St. Louis, MI, USA). Briefly, plasma and brain samples were processed (according to the protocol provided by ELISA Kit instructions) and added into the specific antibody-containing wells in duplicate. Then, the specific detection of antibody-streptavidin-HRP complexes were added, and absorbance was determined using a microplate reader (Multiskan™ SkyHigh, Thermo Fisher Scientific Inc., Waltham, MA, USA). The concentrations of cytokines (pg/ml) were calculated from standard curves obtained by plotting the mean absorbance for each reference standard against each cytokine concentration. For both assays, inter- and intra-assay coefficients of variation were <10%.

2.4. Oxidative stress markers

2.4.1. Thiobarbituric acid reactive substances assay

Due to the ability of thiobarbituric acid to react with lipid peroxidation products (in particular malondialdehyde) accumulating during stressful conditions, lipid peroxides generation in HIP and PFC ($n = 6/\text{group}$) was analyzed by TBARS assay, as previously described (Pasqua et al., 2020; Rocca et al., 2022). In brief, after homogenizing tissues in 0.9% KCl (pH 7.4) (10% w/v), an aliquot of 2 ml of each sample was mixed with 1 ml of 40% (w/v) trichloroacetic acid (TCA) and 1 ml of 0.2% (w/v) 2-thiobarbituric acid (TBA). TBA reagent mixture was also supplemented with 2% (w/v) butylated hydroxytoluene to prevent artificial lipid peroxidation during the assay. The mixture was then heated at 100 °C for 15 min, 2 ml of 70% (w/v) TCA was added, and the samples were centrifuged for 20 min at 3500 rpm. TBARS concentration was estimated spectrophotometrically Multiskan™ SkyHigh (Thermo Fisher Scientific Inc., Milan, Italy) at 523 nm and expressed in nmol/g tissue.

2.4.2. Superoxide dismutase assay

SOD activity was assayed in HIP and PFC ($n = 6/\text{group}$) by measuring the inhibition of pyrogallol auto-oxidation following the method of Marklund and Marklund (1974) as previously reported (Pasqua et al., 2020; Rocca et al., 2022). After being homogenized in a buffer containing 50 mM Tris HCl, (pH 8.2) and 1 mM diethylenetriaminepentaacetic acid, tissues were centrifuged (20 min at 20,000 g). Supernatants were collected and used for SOD activity evaluation, the reaction was initiated by adding 0.2 mM pyrogallol and monitoring its auto-oxidation at 420 nm for 3 min using a Multiskan™ SkyHigh (Thermo Fisher Scientific Inc., Milan, Italy). SOD activity in HIP and PFC was normalized to total protein sample levels, quantified using bovine serum albumin as standard, and was expressed as units per mg of protein considering that 1 U of SOD corresponds to the amount of enzyme, which is able to inhibit the rate of pyrogallol autoxidation by 50%.

2.5. Microbiota analysis

Fecal samples ($n = 6/\text{group}$) were collected in sterile plastic containers and then frozen -80 °C until analysis. In a Stomacher-400 blender, these samples were homogenized. As instructed by the manufacturer, DNA was extracted using a QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain), although samples were combined with the lysis buffer and incubated at 95 °C rather than 70 °C to assure lysis of both Gram-positive and Gram-negative bacteria. A NanoDrop ND-1000 spectrophotometer was used for quantification (Thermo Fisher Scientific, DE, USA). Absorbance ratios were measured for salt, phenol, and protein contamination as well as protein contamination at A260/280 nm and A260/230 nm. The extracted DNA was PCR amplified using the primer pairs, 16S Amplicon PCR Forward Primer:

50TCGTCGGCAGCGTCAGATGTGTATAAGAGA-CAGCCTACGGGNGGCWGCAG, and 16S Amplicon PCR Reverse Primer: 50GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGGACTACHVGGGTATCTAATCC targeting V3 and V4 hypervariable regions of the bacterial 16S rRNA gene (Herlemann et al., 2013). All PCRs were performed in 25 μ l reaction volumes containing 12.5 μ l 2 \times KAPA HiFi Hotstart ready mix (KAPA Biosystems, Woburn, MA, USA), 5 l of each forward and reverse primers (1 μ M) plus 2.5 μ l of extracted DNA (10 ng) under the following cycling conditions: initial denaturation at 95 $^{\circ}$ C for 3 min, followed by cycles of denaturation at 95 $^{\circ}$ C for 30 s, annealing at 55 $^{\circ}$ C for 30 s, and elongation at 72 $^{\circ}$ C for 30 s, with a final extension at 72 $^{\circ}$ C for 5 min. PCR clean-up was performed using AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA) to purify the 16S V3 and V4 amplicon away from free primers and primer dimer species. The next step was to determine the PCR index, in this step dual indexes and Illumina sequencing adapters are added using the Nextera XT Index Kit (Illumina, San Diego, CA, USA). PCR conditions were: 95 $^{\circ}$ C for 3 min; 8 cycles of 95 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 30 s; 72 $^{\circ}$ C for 5 min, and hold at 4 $^{\circ}$ C. The pooled PCR products were purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA) before quantification. The resultant amplicons were sequenced at MiSeq (Illumina, USA), using paired-end (2x300nt) Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA).

Galaxy (Schloss et al., 2009) with the Mothur tool was used for quality assessment of raw reads, which were assembled and screened based on the minimum length of 250 bp and a maximum length of 550 bp. Demultiplexing, quality filtering, length filtering, dereplication, and removal of model organism sequences were all processes in MG-input RAST's processing. The data details and preprocessing settings were as follows: the filtering of FASTQ sequences was done via dynamic trimming. The sequences comprising five bases below the value score (15), which was the lowest Phred quality score specifically described as a high-quality base, were clipped. Relative abundances at the taxonomic levels of Phylum, Family, and Genus were provided for the raw microbiologic data. Microbial community diversity, including α and β diversity, was examined for all experimental groups. By counting the number of readings for each taxon, the observed relative abundance of each taxon was calculated.

3. Statistical analysis

All the data are presented as the mean \pm standard error of the mean (s.e.m.) and were analyzed using SPSS 25.0 software. For the three-chambered social approach, paired *t*-tests were used to compare the time spent sniffing mice and objects as well as time spent in the stranger chamber and the novel object chamber for each group of mice. Two-way ANOVA with Bonferroni's post hoc test was used to compare two variables when $P < 0.05$. For the raw data set, reads of the full processing of amplicons (fastq files) were imported using Quantitative Insights into Microbial Ecology 2 (QIIME 2.0) tools version 2021.4.0, with the raw reads being pre-processed using Cutadapt approach as previously reported (Kechin et al., 2017). Paired-end reads were demultiplexed and featured tables were constructed by using the Divisive Amplicon Denoising Algorithm, which included the following steps: read quality filtering and trimming, error rate estimation, dereplication, read merging plus chimera detection. Taxonomic assignment was obtained using trained sequences (OTUs at 99%) from Silva database version 138 by the q2-feature-classifier QIIME 2 plugin. In order to visualize microbiota composition, stacked bar plots were constructed with ggplot2. Microbiota α -diversity was estimated using Shannon entropy and effective Shannon entropy of the phyloseq package. The β -diversity was generated in a R-vegan package (2.6.0) using the Bray-Curtis distance. Statistical significances between groups were determined by applying the Permutational Multivariate Analysis of Variances (PERMANOVA) for Adonis function of the vegan R-package. Even for this part, *P*-values were corrected for multiple testing by applying the appropriate

indications (Hochberg and Benjamini, 1990) that are applied to compare two variables when $P < 0.05$. For all statistical comparisons, significance was set at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

4. Results

4.1. KD effect on behavioral activities

KD has been shown to be effective in counteracting most abnormal behaviors exhibited by BTBR mice. In particular, C57 group fed with CD spent significantly more time in the stranger chamber (Fig. 1A; *t*-test: $t_{(8)} = 4.352$, $p = 0.003$) in which these mice passed more time exploring stranger mouse rather than novel object (Fig. 1B; *t*-test: $t_{(8)} = 5.440$; $p = 0.001$). Conversely, BTBR mice fed with CD showed an abnormal sociality, as demonstrated by a reduction of time spent in the stranger side (Fig. 1A; *t*-test: $t_{(8)} = 2.174$, $p < 0.05$) and of the time spent interacting with the stranger (Fig. 1B *t*-test: $t_{(8)} = -0.636$, $p = 0.545$). Interestingly, KD treatment was able to completely reverse sociability deficits in these mice, as shown by both the time spent in stranger side (Fig. 1A; *t*-test: $t_{(8)} = 6.290$, $p < 0.001$) and the time exploring stranger rather than the novel object (Fig. 1B; *t*-test: $t_{(8)} = 21.775$, $p < 0.001$). Similarly, KD was also responsible for a greater sociability in C57, as shown by an even greater time spent in the stranger chamber (Fig. 1A; *t*-test: $t_{(8)} = 9.615$; $p < 0.001$) and time interacting with stranger (Fig. 1B; *t*-test: $t_{(8)} = 23.064$, $p < 0.001$). A sociability alteration was also indicated a SI for exploration time that resulted to be notably evident for strain per diet interaction ($F_{(1, 28)} = 12.218$, $p = 0.002$). From the analysis of simple main effects, it was possible to observe a significantly elevated SI for BTBR mice fed with KD with respect to mice that received CD (Fig. 1C; $p < 0.001$). This result was further confirmed by a complete inverse situation of BTBR CD group, which provided a statistically significant lower SI (Fig. 1C, $p < 0.001$) with respect to C57 CD mice. In the case of both C57 mice groups, there were no significant differences of SI variation between the two treatment groups.

As far as total self-grooming duration for repetitive behavior assessment was concerned, a significant interaction between strain and diet ($F_{(1, 28)} = 16.709$; $p < 0.001$) was reported. In particular, time spent explicating self-grooming was extremely reduced in BTBR KD mice when compared to BTBR CD group (Fig. 1D; $p < 0.001$). This was largely noticeable if we consider that self-grooming of BTBR CD mice resulted to be substantially higher with respect to the C57 CD (Fig. 1D; $p < 0.001$). No difference was detected between C57 fed with CD and KD.

To evaluate the effects of a KD diet on anxiety-like behaviors, mice were also tested in the light-dark maze, in which no strain plus diet interactions were reported to spend more time in the light area (Fig. 2A; $F_{(1, 28)} = 0.06768$, $p = 0.797$) together with the total number of transitions (Fig. 2B; $F_{(1, 28)} = 0.5462$, $p = 0.466$). In this case, the diet was not statistically significant nor for time spent in light area (Fig. 2A; $F_{(1, 28)} = 0.06924$, $p = 0.794$) neither for the total number of transitions (Fig. 2B, $F_{(1, 28)} = 1.026$, $p = 0.32$), despite a significantly main effect of strain being detected only for total number of transitions ($F_{(1, 28)} = 5.413$, $p = 0.0320$). However, a significant interaction effect between diet and strain was observed for the first latency to dark area (Fig. 2C; $F_{(1, 28)} = 5.662$, $p = 0.02$). For this part, a significantly higher time of latency to dark area was observed for BTBR fed with KD as compared to BTBR fed with CD (Fig. 2C; $p < 0.01$). Similarly, for C57, KD treatment significantly incremented time spent to enter for the first time in the dark side with respect to the C57 fed with CD (Fig. 2C; $p < 0.001$).

NOR assay was performed to assess short-term memory ability in mice (Fig. 2D). In this case, the interaction effect between strain and diet on DI was statistically significant ($F_{(1, 28)} = 12.44$, $p = 0.001$) as shown by KD treatment causing a higher and positive DI with respect to the BTBR CD (Fig. 2D; $p < 0.001$). Such a relationship goes in the opposite direction for BTBR fed with CD since this diet accounted for a significant lower DI with respect to C57 fed with this same diet (Fig. 2D; $p < 0.001$). Moreover, a moderate improvement in memory of C57 mice fed with KD

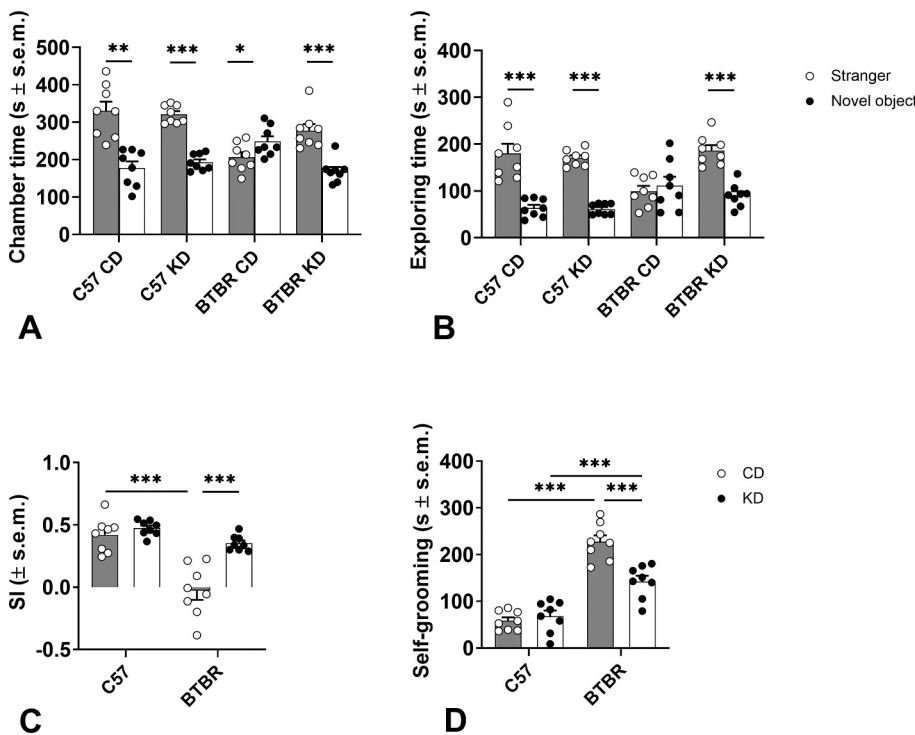


Fig. 1. Effects of KD administration on sociability and repetitive behaviors. Time (s ± s.e.m.) spent in the chamber with the stranger/novel object (A) and exploring the social stimulus (B), along with Sociability Index (SI; C) were evaluated in the Three Chamber Test for both BTBR and C57 mice fed with controlled (CD) or ketogenic (KD) diet (n = 8/group). Time (s ± s.e.m.) spent in repetitive behaviors (D) was evaluated during a Self-Grooming Test for the above mice. Data were statistically evaluated performing paired *t*-tests (A, B) and two-way ANOVA with Bonferroni's post hoc test (C, D) to compare two variables when *P* < 0.05 as reported in Materials and Methods. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

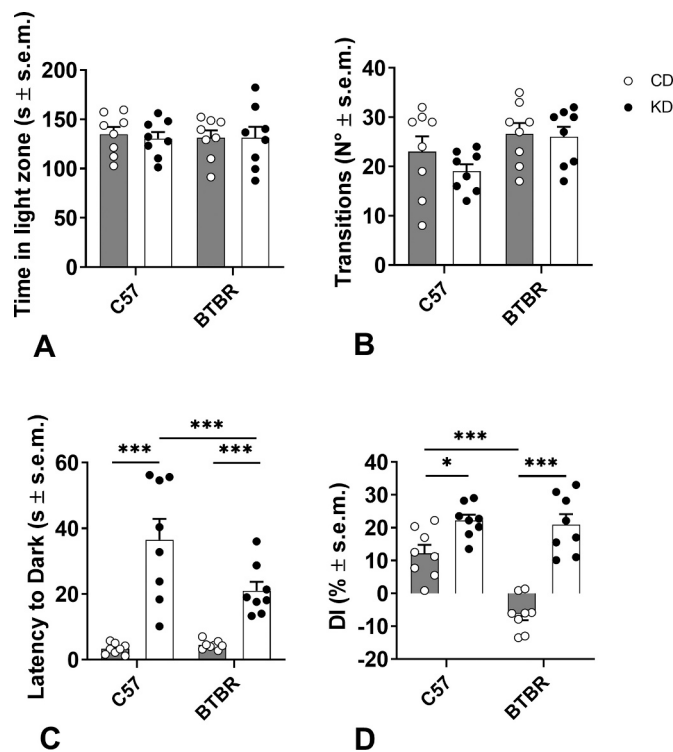


Fig. 2. Effect of KD administration on anxiety like-behaviors and short-memory. Time (s ± s.e.m.) spent in the light zone (A) and the first latency to cross to the dark area (C), along with the number of transitions between the light and dark compartments (B) were evaluated in the Light-Dark Box Test for both BTBR and C57 mice fed with controlled (CD) or ketogenic (KD) diet (n = 8/group). Recognition ability was evaluated as a Discrimination Index (DI) (% ± s.e.m.; D) in the Novel Object Recognition Test. Data were statistically evaluated performing a two-way ANOVA with Bonferroni's post hoc test to compare two variables when *P* < 0.05 as reported in Materials and Methods. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

(Fig. 2D; *p* < 0.05) was also observed with respect to C57 group fed with CD.

4.2. Effect of KD on pro-inflammatory cytokines

TNF-α, IL-1β and IL-6 levels were analyzed in the brain and plasma of the different experimental groups. In the case of TNF-α, significant values were reported in PFC (diet × strain interaction: $F_{(1, 20)} = 9.265, p = 0.006$; Fig. 3A), in HIP (diet effect $F_{(1, 20)} = 6.079, p = 0.02$; strain effect: $F_{(1, 20)} = 76.47, p < 0.001$; Fig. 3B) and plasma (diet × strain interaction: $F_{(1, 20)} = 9.155, p = 0.007$; Fig. 3C). Treatment of BTBR with KD strongly reduced TNF-α levels in PFC (*p* < 0.01; Fig. 3A) and plasma (*p* < 0.001; Fig. 3C) while a moderate reduction was detected in HIP (*p* < 0.05; Fig. 3B) with respect to BTBR fed with CD.

Significant effects were also detected for IL-1β levels in PFC (Fig. 3D; strain effect: $F_{(1, 20)} = 134.0, p < 0.001$; diet effect: $F_{(1, 20)} = 4.686, p = 0.04$) and plasma (Fig. 3F; diet × strain interaction: $F_{(1, 20)} = 17.269, p < 0.001$). Conversely, only the main effect of the strain ($F_{(1, 20)} = 101.290, p < 0.001$) and not of the diet ($F_{(1, 20)} = 3.276, p = 0.09$), nor interaction ($F_{(1, 20)} = 4.218, p = 0.05$), were reported for HIP (Fig. 3E). Indeed, post hoc analysis pointed to BTBR mice that exhibited higher levels of IL-1β in PFC (Fig. 3D; *p* < 0.001), HIP (Fig. 3E; *p* < 0.001) and plasma (Fig. 3F; *p* < 0.001) as compared to C57. Treatment with KD in BTBR significantly reduced IL-1β levels in PFC (Fig. 3D; *p* < 0.05) and plasma (Fig. 3F; *p* < 0.001) with respect to group of BTBR fed with CD.

Contextually, significant interactions between diet and strain were reported for IL-6 levels in PFC (Fig. 3G; $F_{(1, 20)} = 5.680, p = 0.03$) and HIP (Fig. 3H; $F_{(1, 20)} = 5.261, p = 0.03$). On the contrary, no interaction effect was detected in plasma (Fig. 3I; $F_{(1, 20)} = 2.006, p = 0.172$), despite its significant strain ($F_{(1, 20)} = 92.34, p < 0.001$) and diet ($F_{(1, 20)} = 6.061, p = 0.023$) effects. Treatment with KD in BTBR significantly reduced IL-6 levels in PFC (Fig. 3G; *p* < 0.01), HIP (Fig. 3H; *p* < 0.05) and plasma (Fig. 3I; *p* < 0.05) with respect to the group of BTBR fed with CD.

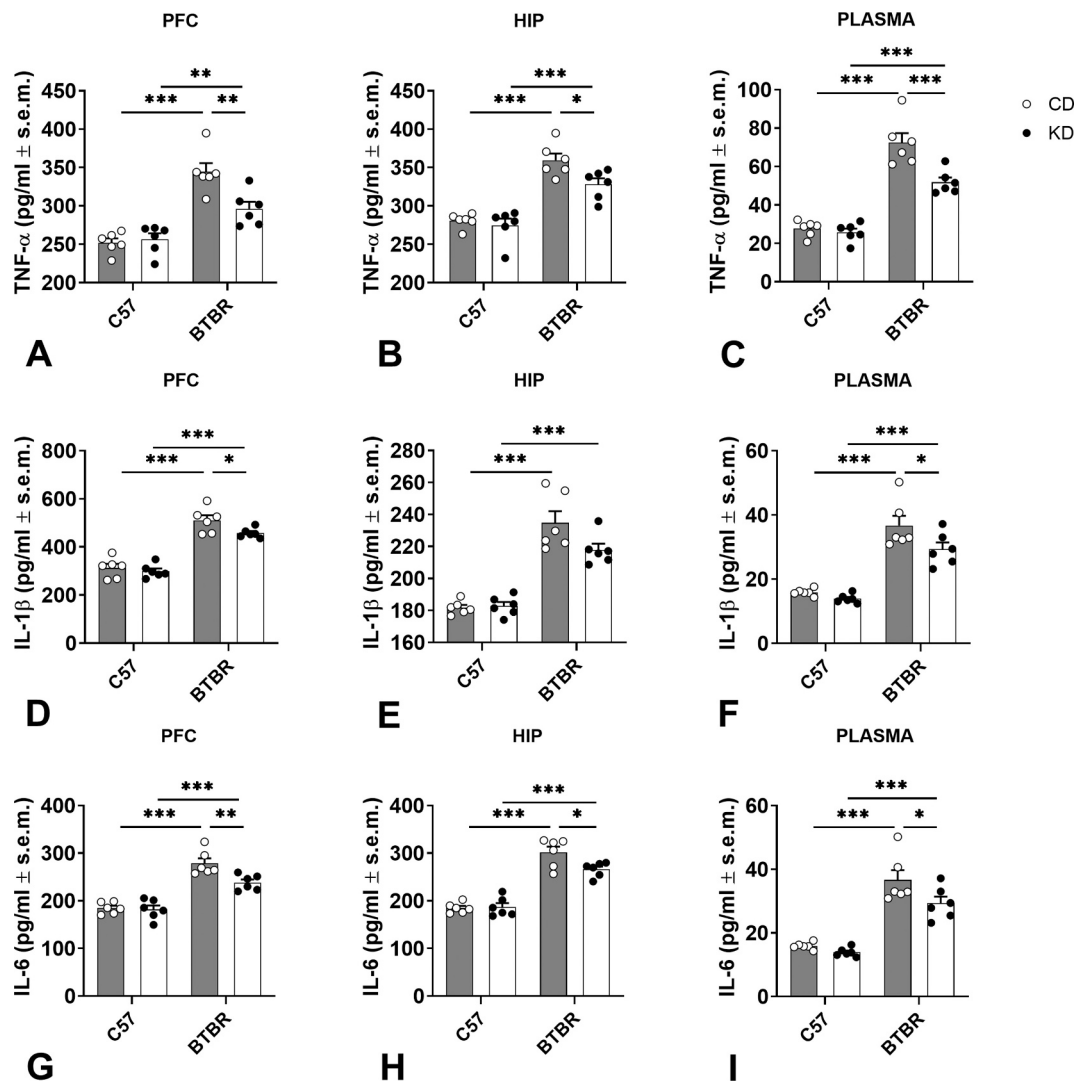


Fig. 3. Effect of KD administration on the levels of cytokines. Data were expressed as pg/ml (\pm s.e.m.) of TNF- α , IL-1 β and IL-6 in PFC (A, D, G), HIP (B, E, H), and the plasma (C, F, I) of both BTBR and C57 mice fed with controlled (CD) or ketogenic (KD) diet ($n = 6$ /group). Data were statistically evaluated performing a two-way ANOVA with Bonferroni's post hoc test to compare two variables when $P < 0.05$ as reported in Materials and Methods. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3. KD and oxidative stress

Regarding oxidative stress conditions, TBARs levels in PFC (Fig. 4A) and HIP (Fig. 4B) demonstrated a significant diet per strain interaction ($F_{(1, 20)} = 5.996$, $p = 0.02$; $F_{(1, 20)} = 5.299$; $p = 0.03$). Elevated TBARs levels were observed in BTBR KD mice when compared to C57 KD in PFC (Fig. 4A; $p < 0.01$) and HIP (Fig. 4B; $p < 0.001$). However, KD treatment in BTBR reduced significantly TBARs levels in PFC (Fig. 4A; $p < 0.05$) and HIP (Fig. 4B; $p < 0.05$) with respect to BTBR fed with CD. In the case of SOD activity, an interaction effect of diet and strain ($F_{(1, 20)} = 5.996$; $p = 0.02$) turned out to be substantially significant in PFC (Fig. 4C). Post hoc analysis demonstrated a significant low SOD activity in PFC of BTBR CD, compared to C57 CD (Fig. 4C; $p < 0.001$), which was incremented after KD treatment (Fig. 4C; $P < 0.05$). No interaction effects ($F_{(1, 20)} = 3.440$; $p = 0.08$) were detected in HIP while, from the analysis of the main activity, only an effect of strain ($F_{(1, 20)} = 34.44$; $p < 0.001$) but not of diet ($F_{(1, 20)} = 3.291$; $p = 0.08$) was reported on SOD activity (Fig. 4D). No difference was instead observed between C57 animals fed with CD and KD for both TBARs levels and SOD activity.

4.4. KD and microbiota

Ecological features of fecal bacterial communities were evaluated in all groups of mice. Significant differences in α -diversity quantified by Shannon entropy and effective Shannon entropy (Fig. 5A, $p < 0.05$) were observed in BTBR CD with respect to C57 CD. No significant difference was observed in α -diversity between both groups of mice fed with KD (Fig. 5A). Rather, treatment with KD resulted in an increment of α -diversity in BTBR with respect to only BTBR fed with CD and in their reduction in C57 with respect to C57 fed with CD. Principal Coordinate Analysis (PCoA) for the β -diversity assessment showed significant differences in the gut bacterial assortment (Fig. 5B), with a clear separation between C57 and BTBR groups fed with CD ($p < 0.001$). Treatment with KD reduced β -diversity between the two strains with BTBR KD samples positioned close to the C57 KD samples (Fig. 5B).

The taxonomic profiles of the fecal microbiome at the Phylum level (Fig. 5C) revealed an increment in the relative abundance of *Actinobacteria* and a decrement in *Proteobacteria* in BTBR KD (0.7%; 0.1%) with respect to BTBR CD (0.01%; 0.2%). The relative abundance of the Phylum *Bacteroidota* accounted for 40% in BTBR CD and 37% C57 CD groups with a modest increment in BTBR KD (52%) and C57 KD (44%). In contrast, the relative abundance of the Phylum *Firmicutes* accounted

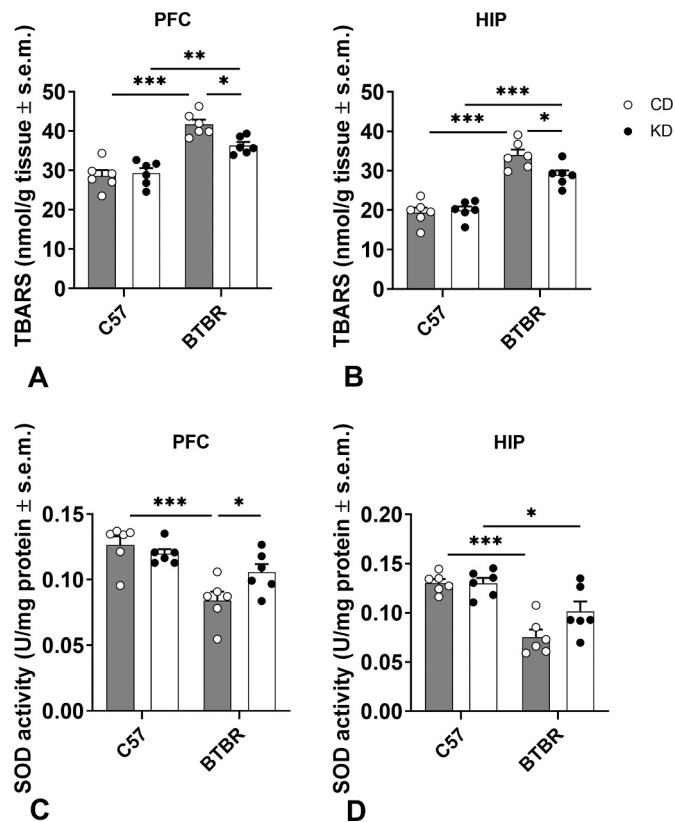


Fig. 4. Effect of KD administration on oxidative stress. Data were expressed as nmol/g tissue (\pm s.e.m.) of TBARS in PFC (A) and HIP (B), and as U/mg protein (\pm s.e.m.) of SOD activity in PFC (C) and HIP (D) of both BTBR and C57 mice fed with controlled (CD) or ketogenic (KD) diet ($n = 6$ /group). Data were statistically evaluated performing a two-way ANOVA with Bonferroni's post hoc test to compare two variables when $P < 0.05$ as reported in Materials and Methods. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

for BTBR CD (60%) and C57 CD (59%) with a modest decrement in BTBR KD (45%) and C57 KD (48%). At the Genus level (Fig. 5D), the abundance of *Lactobacillus* was significantly higher in BTBR CD (44%) with respect to C57 CD (7%) with KD treatment being able to reverse this abnormal elevation in BTBR (5%). Genus *Akkermansia* was significantly more abundant in mice BTBR (1%) and C57 (6%) fed with KD than in BTBR (0.04%) and C57 (0.05%) fed with CD. Similarly, Genus *Blautia* was sharply incremented in BTBR (7%) and C57 (6%) groups fed with KD with respect to BTBR (0.3%) and C57 (0.7%) fed with CD.

5. Discussion

Due to the illusory picture of etiology, a considerable number of ASD patients fails to receive proper behavioral and medical treatments (Baribeau et al., 2022). Among others, KD has emerged as a potential approach to treat different neurological disorders including ASD (Lim et al., 2022), but underlying mechanisms of its action and the adequate intervention period have not been fully established. The present study highlights the value of a 5 week-KD treatment to improve ASD-like behaviors along with mitigating inflammation plus oxidative stress and, contextually, suggesting an important link with the gut microbiota in BTBR mice. Several studies have already shown that BTBR mice display some behavioral features, such as social deficits and repetitive behaviors, resembling the core symptoms of human ASD (Arakawa, 2021; Higuchi et al., 2023). In our case, KD reversed sociability deficits of BTBR since these animals displayed an SI similar to C57 in Three-Chamber Sociability Test. Similarly to our 5-week dietary intervention, others suggested that a 4 week-KD treatment is a promising

therapeutic strategy for prolonged rescue of ASD social deficits via its major product, β -hydroxybutyrate, that could act as an endogenous inhibitor of histone deacetylase so leading to the restoration of histone acetylation in PFC (Qin et al., 2022). In our BTBR mice the excessive self-grooming behavior, which represents a phenotype of restricted repetitive and stereotyped behaviors (Arakawa, 2021), was significantly reduced by KD. These data are consistent with previous results in which KD reduced core-ASD like behaviors (Ruskin et al., 2017), thus reinforcing its role as a potential nutritional approach for behavioral ASD symptoms.

Along with social ameliorations, cognitive performances were also improved in C57 while the deficits were completely reversed in BTBR mice. These findings are in line with studies in which cognitive alterations were mitigated following KD interventions in humans (Desli et al., 2022; Grochowska and Przeliorz, 2022) and rodents (Xu et al., 2022) under different neurological and neurodegenerative conditions. Conversely, reports of increased anxiety in BTBR mice are inconsistent and in some cases controversial depending on the behavioral paradigm used (Chao et al., 2018; Sen et al., 2022), even though these mice typically display increased stress hormones (Meyza and Blanchard, 2017). In the present study, BTBR mice showed no significant difference, in comparison to C57, neither in the time spent in the light chamber nor in the number of transitions, even following KD treatment. Nonetheless, a significant increment was registered for the first latency to dark in BTBR KD, very likely indicating a reduction in their neophobic response. Such a reduction, however, was less evident than C57 thus indicating that our BTBR mice showed a novelty-induced anxiety that resembles food selectivity and neophobia in children with ASD (Rodrigues et al., 2023).

It is important to consider that behavioral abnormalities are frequently linked to inflammatory states and, although inflammation is a first defense mechanism of tissues, an aberrant inflammatory response exacerbates the healing processes and may further aggravate neurological conditions. Indeed, in the course of neurodegenerative illness, chronic neuroinflammation has been observed since pro-inflammatory cytokines, like IL-1 β , IL-6, and TNF- α , are produced and released in large quantities as a result of overactive microglia and astrocytes (Kwon and Koh, 2020). Even in our case, a pro-inflammatory cytokines profile was observed in PCF and HIP as well as in the plasma of BTBR, which is consistent with previous studies (Alsubaiei et al., 2023; Zhao et al., 2021) thus reinforcing their involvement as potential biomarkers for the diagnosis and treatment of ASD. In particular, it has been already demonstrated that these cytokines are linked to protein degradation, mitochondrial dysfunction, defects in axonal transport, and apoptosis, all of which have a negative impact on neuronal functions so interfering with pathways associated with sociability and cognition (Kanellopoulos et al., 2020; McFadden et al., 2020). Additionally, since immune cells and cytokines breach the blood-brain barrier in response to neuroinflammation, they enter the nervous system and speed up neuroinflammation and neurodegeneration (Takata et al., 2021). Interestingly, administration of KD reduced the levels of IL-1 β , IL-6, and TNF- α in both brain areas and plasma of BTBR mice in a similar manner to other probiotics- and prebiotics-enriched diets (Alsubaiei et al., 2023). Such results seem to suggest that even a 5 week-KD treatment could play a crucial role on inflammatory states in ASD, possibly orchestrating a neuroprotection program similarly to other conditions (Sethuraman et al., 2022). This hypothesis is not that so far off since, recently, it has been reported that β -hydroxybutyrate is a promising potential for reducing inflammation in neurodegenerative disorders via the G-protein-coupled receptor 109A and the deactivation of the inflammasome complex in microglia (Jayashankar et al., 2023).

Even oxidative stress is a key feature of many neurological disorders and, specifically, ASD has been linked to an increase in ROS and reduction in antioxidant capacity (Liu et al., 2022a). Accordingly, the results of the present study showed significant increases in TBARS levels together with a decline of SOD activity in both PFC and HIP of BTBR that

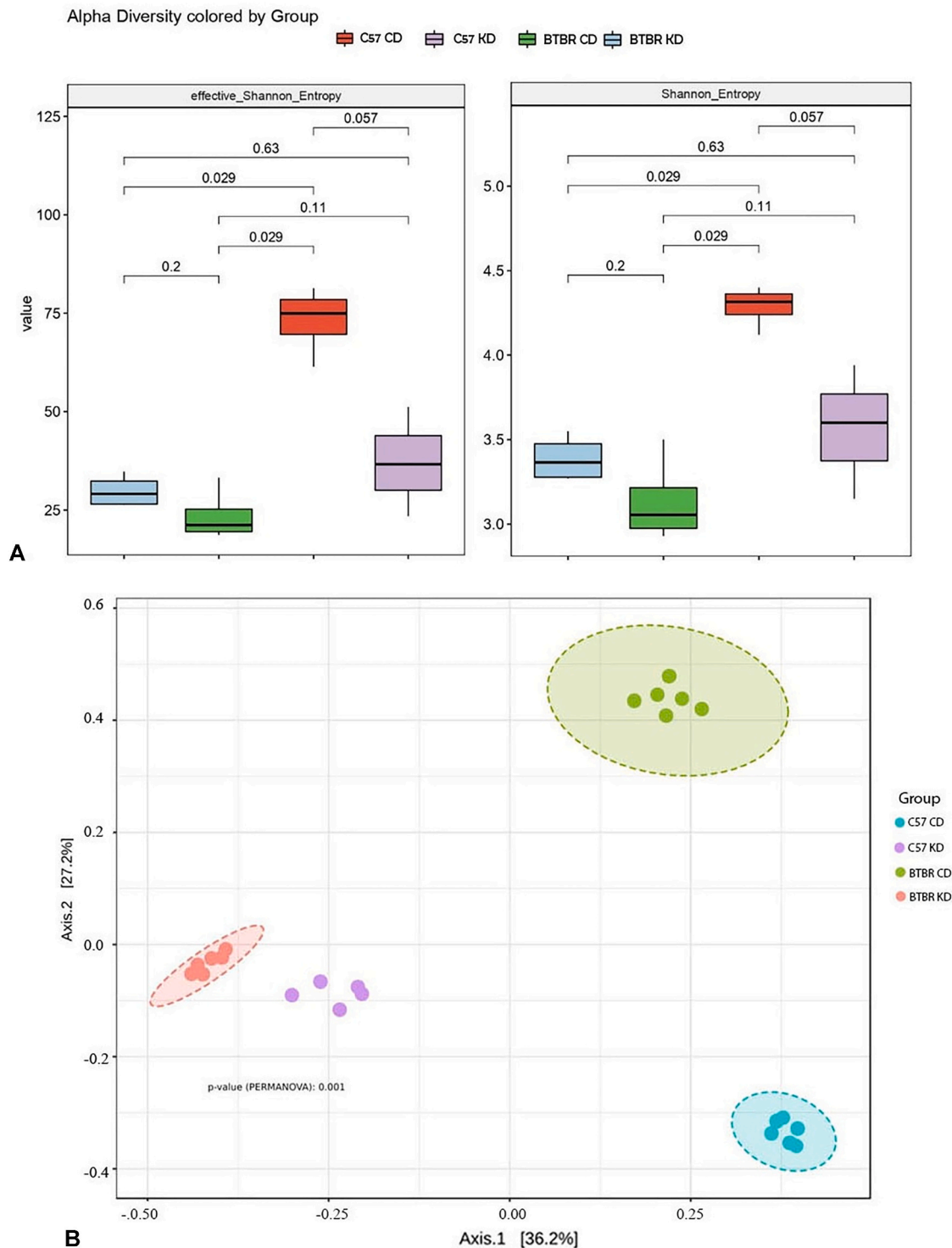


Fig. 5. Effect of KD on gut microbiota. α -diversity was estimated using effective Shannon entropy and Shannon entropy (A) for both BTBR and C57 mice fed with controlled (CD) or ketogenic (KD) diet ($n = 6$ /group). Principal Coordinate Analysis (PCoA) for the β -diversity was handled in the above experimental groups (B). The relative abundance at both Phylum (C) and Genus (D) levels were provided for the raw microbiologic data of the same animals as reported in Materials and Methods.

may turn out to be in activating intracellular signaling pathways responsible for both behavioral alterations and inflammation. Indeed, elevated concentrations of ROS can create vicious cycles, which maintain a high secretion of pro-inflammatory cytokines and chemokines especially in specific brain regions associated with ASD, such as PFC and HIP (Liu et al., 2022a). It is worthy to note that treatment with KD significantly reduced TBARS levels and incremented SOD activity in our

BTBR mice. Such a condition should not be so surprising since it has been already shown that KD can delay the onset of age-related neurodegenerative diseases through a neuroprotective program that includes antioxidant and anti-inflammatory effects (Kovács et al., 2021). Indeed, the most abundant ketone body, β -hydroxybutyrate, inhibits the NLRP3 inflammasome in myeloid cells, a key potentiator of age-related inflammation (Goldberg et al., 2023). Such a KD role has been also

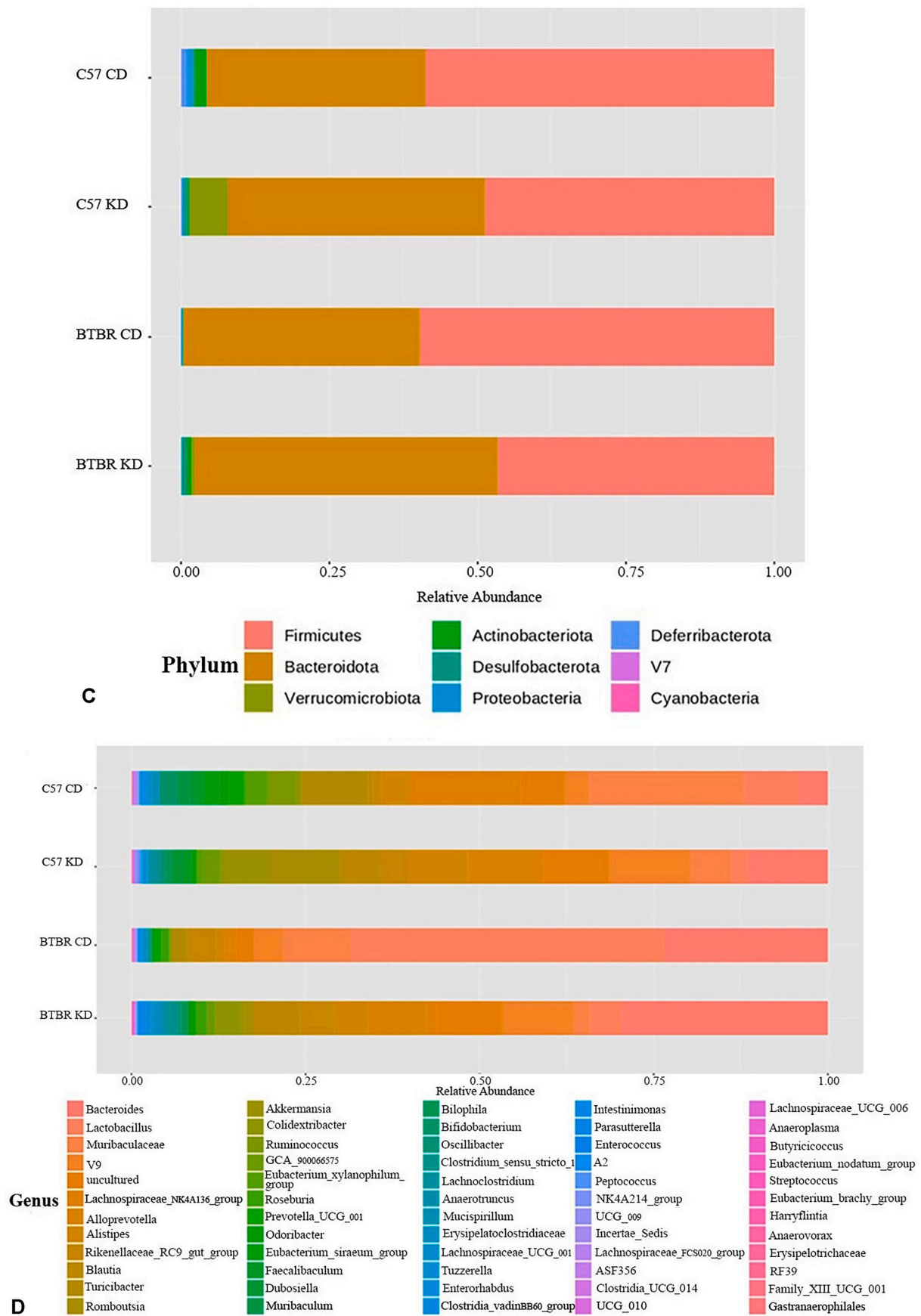


Fig. 5. (continued).

reported for ASD, even though following longer periods of treatment (Li et al., 2021). In this case, KD intervention therapy induces the generation of acetyl-CoA in the mitochondria of the liver, via fatty acid oxidation, thus shunting to the formation of ketone bodies with neuroprotective effects in the brain. Indeed, ketone bodies improve energy metabolism by enhancing ATP production and normalizing mitochondrial function, via mitochondrial biogenesis and reduction of oxidative stress, which in turn reduces neuronal death. In addition, they regulate neurotransmitters and inhibit activation of the mammalian target of rapamycin (mTOR) signaling pathway as well as modulating the gut microbiota composition.

In reference to the latter condition, a 5-week KD intervention proved to be useful for the induction of an important modulation of the gut microbiota composition of our BTBR mice. In particular, KD slightly increased α -diversity in BTBR thus partially remodeling dysbiosis that is frequently accompanied by a decreased microbiota diversity (Kim et al., 2020). In any case, it appeared that KD has enhanced beneficial bacterial hosts as shown by a rapid increase in *Akkermansia* and *Blautia*. Additionally, KD altered β -diversity in both strains and greatly decreased their clustering separation, demonstrating its capacity to change the biological community structures in the gut. Interestingly, taxonomic study showed that *Lactobacillus* levels were abnormally higher in BTBR rather than in C57 mice, similarly to others (Liu et al., 2022b), but this abnormality was completely abolished by KD therapy. *Lactobacillus* species are frequently used as probiotics and have a great number of health advantages. However, an exaggerated abundance of *Lactobacillus* has been reported in both ASD patients and animal models (Alamoudi et al., 2022; Avolio et al., 2022), and some of its strains were associated to oxidative stress and negative immune effect (Lee et al., 2021; Zegarra-Ruiz et al., 2019). Surprisingly, it has been previously observed that mice treated with KD demonstrated enhanced abundance of *Lactobacilli* by 3.2 times with respect to healthy animals (Kaviyarasan et al., 2022). On this basis, it is evident that the function of *Lactobacillus* is still debatable and further investigations are required to clarify the effects of its strains in the gut-brain axis of ASD and especially following KD treatment. Concomitantly, KD increased the relative abundance of putatively beneficial microbiota (*Akkermansia* and *Blautia*) in both BTBR and C57. For instance, it has been demonstrated that administration of a live biotherapeutic strain, i.e. *Blautia stercoris* MRx0006, is capable of improving behavioral outcomes relevant to ASD, including sociability and stereotypical behaviors (Sen et al., 2022). Moreover, it appeared that after KD the cognitive decay was halted, and spatial memory was improved by increasing kynurenic acid production with the help of *Akkermansia* (Kaviyarasan et al., 2022). *Akkermansia* was also reported to relieve systemic inflammation by decreasing the concentration of pro-inflammatory markers together with producing beneficial short-chain fatty acids, predominantly acetate and propionate, which were shown to be vital for gut and neuronal communication as well as homeostasis (Akhtar et al., 2022).

In conclusion, KD has shown to be a multifunctional approach in ASD, supporting peripheral and cerebral anti-inflammatory effects through a reduction of key pro-inflammatory cytokines and antioxidant plus anti-lipoperoxidative capabilities, along with a modulation of the gut-brain axis by remodeling intestinal microbiota composition. However, we are now at the beginning since there are still insufficient studies to deduce KD effect on the gut microbiota, its therapeutic effectiveness as well as the underlying mechanism of action. Future studies, using different experimental paradigms (duration, probiotic supplements, etc.), plus evidence on the possible pathways and specific molecular factors are recommended to examine the effects of this dietary intervention on gut microbiota and the associated therapeutic implications. It would be desirable to monitor the long-term beneficial effects and potential adverse events of KD. In addition, it is necessary to evaluate intestinal inflammation, endothelial integrity, and metabolomic markers. In any case, there is little doubt that the potential protective and preventative qualities of KD are enormous, given the direct modulatory

impact it has on microglia for reducing inflammation in neurodegenerative disorders (Jayashankar et al., 2023).

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CRediT authorship contribution statement

Iliaria Olivito: Conceptualization, Investigation, Writing – original draft, Visualization. **Ennio Avolio:** Conceptualization, Investigation, Project administration. **Damiana Minervini:** Investigation, Visualization. **Teresa Soda:** Investigation. **Carmine Rocca:** Validation, Investigation. **Tommaso Angelone:** Validation, Investigation. **Francesco Salvatore Iaquinta:** Formal analysis. **Dina Bellizzi:** Validation, Investigation. **Francesco De Rango:** Formal analysis, Investigation, Visualization. **Rosalinda Bruno:** Resources. **Loredana De Bartolo:** Resources. **Raffaella Alò:** Conceptualization, Validation, Project administration. **Marcello Canonaco:** Conceptualization, Writing – original draft, Supervision. **Rosa Maria Facciolo:** Conceptualization, Validation, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that there is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

Data availability

Data will be made available on request.

Acknowledgements

This paper is dedicated to the memory of Prof. Marcello Canonaco who passed away on Monday October 3rd, 2022. We will remember Marcello forever not only for his scientific and academic expertise, but also for having spread rigorous scientific research, always opened to the contribution of different disciplines and cooperation between colleagues. Above all, Marcello has been generous, kind, an extraordinary mentor.

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