Research Article

Neonatal Gut Microbiota Disruption Increases Myelin Damage and Neurobehavioral Impairments in Cuprizone-Induced Toxic Model

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Abstract | Multiple sclerosis (MS) is a chronic neuroinflammatory, autoimmune, and neurodegenerative disorder of the central nervous system, characterized primarily by immune-mediated damage to the myelin sheath. Although its exact origin remains unknown, oxidative stress and inflammatory cascades are key contributors to disease progression. Recent research has increasingly demonstrated that gut microbiota plays a critical role in brain development and immune regulation, with dysbiosis implicated in several hallmark features of MS, including inflammation, myelin damage, and impaired repair. Aims: This study aimed to investigate the effects of gut microbiota depletion induced specifically during the critical early-life developmental window on myelin damage and repair in adulthood, using the cuprizone-toxic model of MS in Swiss albino mice. To achieve this, we depleted the gut microbiota of newborn mice with broad-spectrum antibiotics, following normal development into adulthood; myelin damage was induced with cuprizone. We then assessed motor and affective behavioral outcomes, oxidative stress markers, as well as demyelination and remyelination histological assessment. Antibiotic-induced microbiota depletion significantly worsened anxiety- and depression-like behaviors, memory deficits, and motor impairments, with only partial recovery during remyelination. This depletion was associated with sustained elevations in nitric oxide and lipid peroxidation and a paradoxical decrease in catalase activity across key brain regions. Histological analysis revealed exacerbated demyelination and impaired remyelination in the corpus callosum following antibiotic treatment. Early-life gut microbiota depletion during a critical developmental window exacerbates behavioral deficits, and oxidative stress, and impairs myelin repair following demyelinating injury. These findings underscore the essential role of a healthy gut microbiota in supporting neuroprotection and efficient remyelination, suggesting that disruptions in microbial communities during development may increase vulnerability to neurodegenerative and neuropsychiatric disorders.

Keywords | Multiple sclerosis, Gut microbiota, Dysbiosis, Antibiotics, Cuprizone model, Behavioral assessment, Oxidative stress, Biochemicals markers, Demyelination, Remyelination

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INTRODUCTION

Multiple sclerosis (MS) is a chronic, progressive inflammatory disease of the central nervous system (CNS), characterized by immune-mediated damage to the myelin sheath, leading to demyelination and subsequent axonal injury (Dobson and Giovannoni, 2019). This myelin damage disrupts nerve signal transmission and underlies

the diverse symptoms of MS (Maciak *et al*., 2023). Despite extensive research, the etiology of MS remains poorly understood, it is believed to result from a complex interplay of genetic and environmental factors (Alfredsson and Olsson, 2019; Zarghami *et al*., 2021).

The cuprizone (CPZ) model is a well-established experimental system used to study demyelination and remyelination in the CNS (Kipp *et al*., 2017), closely mimicking aspects of MS. In this model, mice are fed a diet containing CPZ, which selectively induces oligodendrocyte death and subsequent demyelination, particularly in the corpus callosum (CC), spontaneous remyelination occurs following CPZ withdrawal (Murayama *et al*., 2025), suggesting that the CPZ model is particularly valuable for evaluating potential therapeutics that can both prevent demyelination and promote remyelination (Wang and Kasper, 2014).

The gut microbiota has recently emerged as a critical environmental factor influencing MS risk and progression (Nourbakhsh and Mowry 2019; Altieri *et al*., 2023). The gut microbiome plays a vital role in maintaining immune homeostasis, and its perturbation -known as dysbiosis- has been linked to increased susceptibility to autoimmune and neuroinflammatory disorders, including MS (Freedman *et al*., 2018; Shahi *et al*., 2022). Studies have shown that individuals with MS exhibit significant alterations in their gut microbiota composition compared to healthy controls (Plassais *et al*., 2021). These microbial changes are associated with a pro-inflammatory immune environment that may trigger or exacerbate CNS autoimmunity and demyelination (Cekanaviciute *et al*., 2017). Supporting this, gut microbiota from MS patients can modulate immune responses and worsen disease severity when transferred to mice (Berer *et al*., 2017), highlighting a functional connection between gut dysbiosis and CNS demyelination.

To further explore this relationship, most studies investigating the link between gut dysbiosis and CNS demyelination have focused on the inflammatory experimental autoimmune encephalomyelitis model (Berer *et al*., 2017; Correale *et al*., 2022), which mimics autoimmune pathology. In contrast, research using the non-inflammatory CPZ model remains very limited. Among these few CPZ studies, some reported that antibiotic-induced dysbiosis exacerbates CPZ-induced demyelination and alters microglial activity-for instance, oral administration of broad-spectrum antibiotics (Abx) significantly worsened demyelination (McMurran *et al*., 2019), while others find that chronic Abx treatment, despite effectively depleting the microbiome, does not significantly affect demyelination or remyelination (Murayama *et al*., 2025).

Although gut microbiota depletion using broad-spectrum antibiotic cocktails has been used in the CPZ-induced demyelination model (McMurran *et al*., 2019; Murayama *et al*., 2025), these studies were conducted on adult animals, whereas investigations targeting the effects of microbiota depletion during early life and its subsequent impact on adult susceptibility to CPZ-induced demyelination and remyelination remain very limited. Hence, our study aimed to fill this knowledge gap by evaluating how depletion of the neonatal gut microbiota using broad-spectrum antibiotics affects neurological aspects, including behavior, biochemistry, and histological outcomes such as myelin degradation and repair, in adult mice subjected to CPZ intoxication. This approach addresses a novel aspect of the gut-brain axis role in MS pathogenesis by focusing on early developmental microbiota influences on CNS vulnerability and recovery.

MATERIALS AND METHODS

Experimental animals

A total of 10 pregnant Swiss Albino female mice were initially used in this study, obtained from our animal housing facility (Ibn Tofail University, Morocco), where they were born and raised. Animals were individually housed in standard cages under controlled environmental conditions: a 12h dark/light cycle (lights on from 6:00 PM to 6:00 AM), temperature maintained at 24˚c ±1˚, and appropriate humidity. Food and water were provided ad libitum.

After excluding invalid pups (sick or weak), approximately 36 healthy offspring were retained. Following weaning, the newborns were randomly assigned to three experimental groups (detailed in the next section). The animals were then marked to facilitate identification and monitoring. The day of birth is referred to as PN1. Efforts were made to maintain a balanced sex ratio across the groups.

All experimental protocols were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the International Animal Care and Use Committee (IACUC) guidelines. The study was approved by the Local University Ethics Committee for animal experiments at Ibn Tofail University.

Experimental protocol

To fulfill the aim of our study, three experimental groups were used.

- The control group (Vehicle): Untreated animals fed standard rodent chow and provided with normal drinking water, which served as the vehicle.
- The CPZ group: Mice received a diet containing CPZ mixed with standard rodent chow.

- The neonatal antibiotic + CPZ group (Abx + CPZ): Mice exposed to antibiotics during lactation (PN10–PN21) and subsequently to a CPZ diet.

To deplete the offspring's gut microbiota, dams were administered a broad-spectrum Abx from PN10 until weaning at PN21. The antibiotic cocktail, consisting of amoxicillin, clarithromycin, and metronidazole 75 mg/kg/day each: The Abx solution was prepared fresh daily. To prevent the re-establishment of the microbiota, animals were closely monitored, and bedding was changed every two days. Regular weighing allowed for adjustment of treatment intake.

At PN28, mice were administered CPZ (Oxalic acid bis, 98%, Thermo Scientific, Cat No 154420250-25gr) mixed daily with standard rodent chow to a concentration of 0.5%, for three weeks to induce the acute phase of demyelination. Afterward, mice were returned to a normal chow diet for two weeks to allow remyelination.

The experimental timeline was structured to assess both demyelination and remyelination phases as shown in Figure 1. After three weeks of CPZ exposure (PN28–PN49), a subset of animals underwent behavioral testing and subsequent euthanasia starting at PN50 to evaluate the demyelination phase. The remaining animals were fed a standard diet from PN49 onwards to allow remyelination, then underwent behavioral testing and were euthanized from PN60 onwards to assess their recovery.

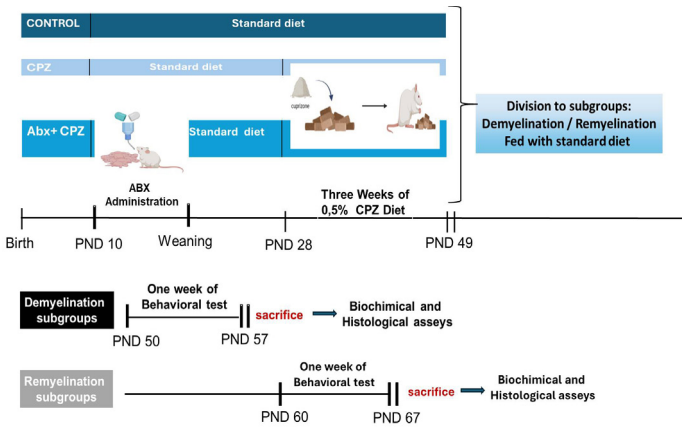

Figure 1: Experimental timeline showing neonatal antibiotic administration, cuprizone (CPZ) treatment, and behavioral/histological assessments during demyelination and remyelination phases.

Following the behavioral assessments, animals were euthanized by cervical dislocation, and brains were rapidly collected for biochemical and histological analyses.

Neurobehavioral tests

In this study, five standardized behavioral tests were used to assess anxiety-like behavior, depression-like behavior, memory, and motor function. The Open Field Test (OFT) and Tail Suspension Test (TST) assessed anxiety-like behavior and depression-like behavior, respectively. Recognition memory was measured using the Novel Object Recognition test (NOR test). Motor coordination and neuromuscular strength were evaluated with the Rotarod and Hanging Wire tests (HWT). Each test included an initial phase of habituation to allow animals to acclimate to the apparatus, followed by a test session conducted 24 hours later to reduce stress-related variability. Testing took place over one week in a dedicated, controlled environment with consistent lighting, noise levels, and temperature to ensure reliable results. Throughout all behavioral assessments and subsequent video analyses, experimenters were blinded to the group assignments to minimize observed bias and enhance the objectivity of the results.

Anxiety-like measurement

The OFT is a widely used test to assess anxiety-like behavior in rodents (Zghari *et al*., 2023; El-Hamzaoui *et al*., 2024). The test was conducted in a rectangular arena (40 x 40 cm) enclosed by walls (30 cm high) to prevent the animals from escaping. The arena is divided into central and peripheral zones, with a light source placed above the apparatus. At the start of the test, the animals were placed in the center and allowed to freely explore the new environment for 10 minutes. Meanwhile, the examiner monitored and recorded the rodent's behavior using a camera to measure parameters that provide insight into anxiety levels, such as time spent in the center versus the periphery and total distance traveled. To prevent olfactory cues from influencing behavior, the apparatus was cleaned with alcohol between each trial.

Depression-like measurement

The TST is a behavioral assay used to evaluate depression-like behavior in rodents (Can *et al*., 2011), particularly mice. In this test, each mouse was suspended by its tail for five minutes, and its movements were recorded. Freezing periods were measured and interpreted as behavioral despair.

Learning and memory assessment

To evaluate recognition memory in mice, we used the NOR test, as explained by Lueptow (2017). During this test, mice were first allowed to explore two identical objects in an arena during the familiarization phase. After a delay, the examiner replaced one of the old objects with a new one, and the animals were returned to the arena for the test phase. The time spent exploring the novel object compared to the familiar one was measured. This parameter is commonly used to assess recognition memory and cognitive function.

Motricity and coordination assessment

The rotarod test

This test is one of the most commonly used and well-established tests for assessing motor coordination and balance in rodents. The Rotarod is considered a standard tool in preclinical research because of its simplicity, reliability, and sensitivity to motor deficits caused by neurological disorders, drug effects, or injuries. During the test, animals are placed on a rotating rod, and their mobility time is recorded. To ensure safety, the mice land on a cushioned surface beneath the rod. Before testing, the mice are trained by first balancing on a stationary rod, followed by practice on a continuously rotating rod, according to the protocol described by Lubrich *et al.* (2022).

The hanging wire test

The HWT was used to assess muscle strength, endurance, and motor coordination in rodents (Hoffman and Winder 2016). In this test, each mouse was placed on a thin horizontal wire, grasping it with its forelimbs. The time the animal managed to hang (the hanging time) before falling was recorded as a measure of neuromuscular function. To ensure safety, a cushioned surface was placed beneath the wire.

Biochemical analyses

Tissue preparation

Upon the completion of the behavioral tests, mice intended for biochemical analysis were sacrificed by cervical translocation. The three targeted brain structures; prefrontal cortex (PFC), hippocampus (HPC), and cerebellum (CB) were rapidly extracted and dissected separately. Tissue samples were homogenized in ice-cold phosphate buffer (50 mM, pH: 7.4) at a volume of 10 mL/g of tissue using a homogenizer. Subsequently the homogenates were centrifuged at 3000 x g for 30 minutes at 4°C to preserve protein integrity, and the resulting supernatants were carefully aliquoted and stored at -80 °C (Brikat *et al.*, 2024; Nassiri *et al.*, 2024).

Lipid peroxidation (LPO) assay

In this study, the formation of oxidized lipids was evaluated in three targeted brain structures (PFC, HPC, and CB) by measuring ThioBarbituric Acid Reactive Substances (TBARS), a marker of lipid peroxidation. A mixture was prepared by combining tissue homogenates with 10% trichloroacetic acid and 0.67% thiobarbituric acid. The resulting solution was placed in a water bath maintained at 90°C for 15 minutes. Afterwards, butanol was added in a 2:1 (v/v) ratio, followed by centrifugation at 1000 x g for 10 min. The absorbance of the supernatant was then measured spectrophotometrically at 535 nm to determine TBARS levels (Benmhammed *et al.*, 2024; Azirar *et al.*, 2025).

Nitric oxide (NO) assay

To measure NO levels in biological samples, nitrite concentration is widely accepted as an indicator of its activity. NO undergoes auto-oxidation in aqueous solution, favoring nitrite as the stable end-product rather than nitrate (Berkiks *et al.*, 2018). Hence, we quantified nitrite levels in three brain structures (PFC, HPC, and CV) using the Griess reaction based on diazotization. Equal volumes of samples and Griess reagent were mixed and incubated at room temperature for 30 min, after which absorbance was measured at 540 nm. Tissue NO levels were calculated as µmol/g of tissue (Rezqaoui *et al.*, 2023; Ibouzine-Dine *et al.*, 2024).

Antioxidant catalase (CAT) assay

To assess catalase activity in the targeted brain structures, we followed the protocol described by Aebi (1984). We mixed 50 µl of the sample supernatant with 1.95 ml of 50 mM phosphate buffer (pH = 7.4) in a cuvette. To initiate the reaction, 1.0 ml of the freshly prepared 50 mM H_2O_2 was added. The decomposition of H_2O_2 was then monitored spectrophotometrically by measuring the absorbance at 240nm over 3 min. Results were expressed as international unit's IU/g of tissue. One unit of catalase was defined as the amount of enzyme required to degrade one µmol of H_2O_2 per minute under standardized conditions (25°C).

Luxol fast blue staining (LFB) for myelination assessment

Tissue preparation and staining protocol

Mice designated for histological analysis were sacrificed by cervical dislocation. They were then transcardially perfused with 30 ml of ice-cold phosphate-buffered saline (PBS), followed by the same volume of 4% paraformaldehyde (PFA) in PBS for tissue fixation (Hobro and Smith 2017). After perfusion, brains were carefully extracted and preserved in 4% PFA at room temperature for 24 hours. The tissues were subsequently immersed in a 30% sucrose solution to enhance structural integrity and facilitate sectioning. The brains were sliced using a vibratome (VT1000S Leica Microsystems AG, Germany) into coronal sections of 30 µm thickness. Sections containing the corpus callosum were mounted on gelatin-coated slides, air-dried, and then stained with LFB according to the Kluver-Barrera protocol (Joshua, 1988) to visualize myelin in the CNS.

Image analysis

After mounting the sections of interest, imaging was performed using a photomicroscope (B-382 Pli-ALC, Optika, Italy, with a P8 Pro camera) (El-Marzouki *et al.*, 2025). For each experimental group, four images were captured at two magnifications, x4 and x10. This approach

was designed to comprehensively sample the corpus callosum (CC) and provide robust data for subsequent statistical evaluation.

Regions corresponding to the CC were manually outlined based on established anatomical landmarks using ImageJ software (version 1.54g).

Before analysis, images were calibrated to convert pixels to square microns for precise quantification of stained areas. To distinguish myelinated from demyelinated regions objectively, Huang's fuzzy thresholding algorithm (an automated image thresholding technique) was applied to RGB images. Threshold values were set using preliminary images and consistently applied to all samples for uniform segmentation. Demyelination percentage was calculated as: Demyelination % = (Demyelinated Area / Total CC Area) × 100%.

Statistical analysis

All analyses were conducted using SPSS version 29 (IBM Corp., Armonk, NY, USA), and graphs were made using Prism 8 (GraphPad Software Inc., San Diego, 189 CA, USA). Data were assessed for normality and homogeneity of variances prior to analysis (El-Hamzaoui *et al*., 2024). Differences among groups were evaluated using a two-way analysis of variance (ANOVA), followed by Sidak's multiple comparisons test for post hoc analysis. All data were presented as means ± standard error of the mean (SEM). A p-value of less than 0.05 was considered statistically significant.

RESULTS

Behavioral tests

Anxiety-like Behavior assessment

Figure 2 shows the results of anxiety levels measured in the OFT. During demyelination, the total time spent in the center of the apparatus was significantly reduced for the CPZ group (17s, $p < 0.001$) and the Abx + CPZ (23s, $p < 0.01$) compared to the controls (29s). A significant difference was also observed between the CPZ and Abx+ CPZ groups ($p < 0.05$). Regarding remyelination, a clear improvement was observed in the time spent in the center for both CPZ ($p < 0.001$) and Abx + CPZ ($p < 0.01$) groups, reaching levels comparable to the control group.

Depression-like behavior assessment

To assess depression-like behavior we used the TST, as shown in Figure 3. The results of this test revealed a clear increase in freezing time in the Abx + CPZ group (80s, $p < 0.001$) compared to both the control (6.9s) and CPZ (10s) groups, with the CPZ group also exhibiting a relative increase ($p < 0.05$) to controls during demyelination. Notably, freezing time in the Abx + CPZ group improved markedly during the remyelination phase (45s, $p < 0.001$); however, it remained substantially higher compared to the other two groups ($p < 0.001$). It is important to note that the Freezing time in the CPZ group during remyelination was nearly identical to that of the control group.

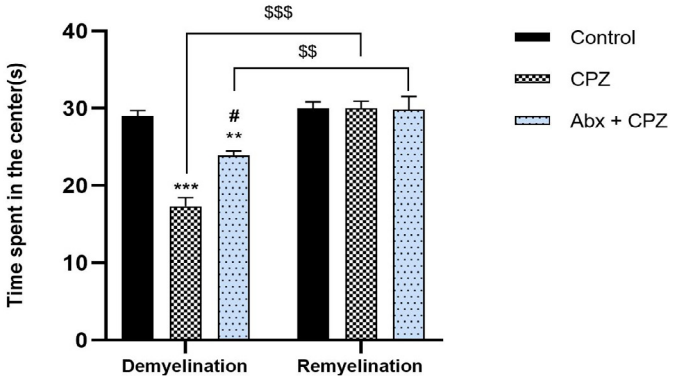

Figure 2: Open Field Test (OFT) results showing time spent in the center zone during demyelination and remyelination in the cuprizone (CPZ)-induced toxic Model. Abx: broad-spectrum antibiotics. The data are presented as mean ± S.E.M. $p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. CPZ treated group; $^{\$}p < 0.05$ for comparisons between the demyelination and remyelination phases.

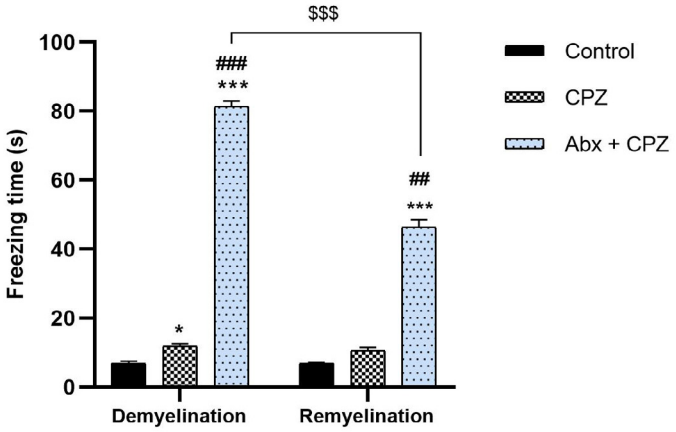

Figure 3: Tail Suspension Test (TST) results quantifying freezing time as a measure of depression-like behavior during demyelination and remyelination in the Cuprizone (CPZ)-induced toxic Model. Abx: broad-spectrum antibiotics. The data are presented as mean ± S.E.M. $p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. CPZ treated group; $^{\$}p < 0.05$ for comparisons between the demyelination and remyelination phases.

Learning and memory assessment

To evaluate memory, the NOR test was employed, as illustrated in Figure 4. The results revealed a significant decrease in the time spent exploring the novel object zone in the Abx+ CPZ group (21s, $p < 0.001$) compared to both the control (59s) and CPZ (38s) groups. Additionally, the CPZ group showed a notably reduced exploration time

relative to controls during demyelination ($p < 0.01$).

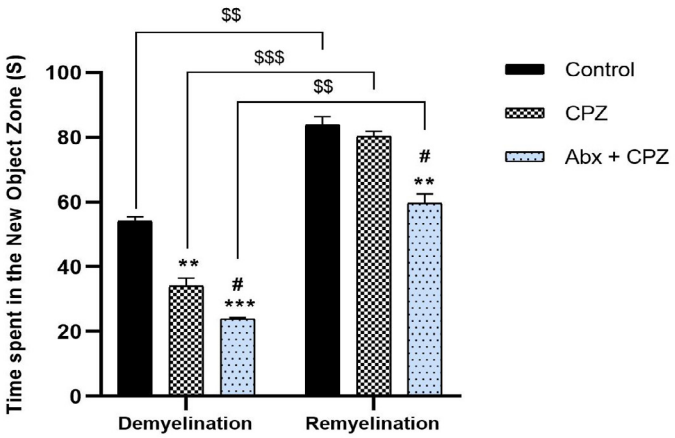

Figure 4: Novel Object Recognition Test (NOR test) results showing time spent in the new object zone during demyelination and remyelination in the Cuprizone (CPZ)-induced toxic Model. Abx: broad-spectrum antibiotics. The data are presented as mean ± S.E.M. $p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. CPZ treated group; $^{\$}p < 0.05$ for comparisons between the demyelination and remyelination phases.

Notably, during the remyelination phase, all groups improved significantly their novel object exploration time ($p < 0.01$), with the CPZ group showing the most pronounced recovery ($p < 0.001$). However, the Abx+ CPZ group's exploration time in the novel object zone (60s) remained significantly lower compared to both controls (82s, $p < 0.001$) and CPZ (79s, $p < 0.01$).

Motricity and coordination assessment

The rotarod test

To evaluate motor coordination and balance, the Rotarod test was used. Our results presented in Figure 5 revealed an important reduction in rotarod mobility time in both the Abx+ CPZ group (13 s) and the CPZ groups (23 s) compared to the control (40 s, $p < 0.001$). Moreover, the performance decrease in the Abx + CPZ group was significantly pronounced not only compared to controls but also relative to the CPZ group ($p < 0.01$).

During the remyelination phase, both the Abx + CPZ and CPZ groups demonstrated significant improvements in rotarod performance ($p < 0.001$). However, despite this positive evolution, the Abx + CPZ group's mobility time (21s) remained significantly lower than that of controls (39s, $p < 0.01$) and the CPZ group (32s, $p < 0.05$).

The HWT

The HWT was also used to assess motor function, coordination, and balance during demyelination and remyelination following neonatal gut microbiota depletion induced by a broad-spectrum antibiotic cocktail.

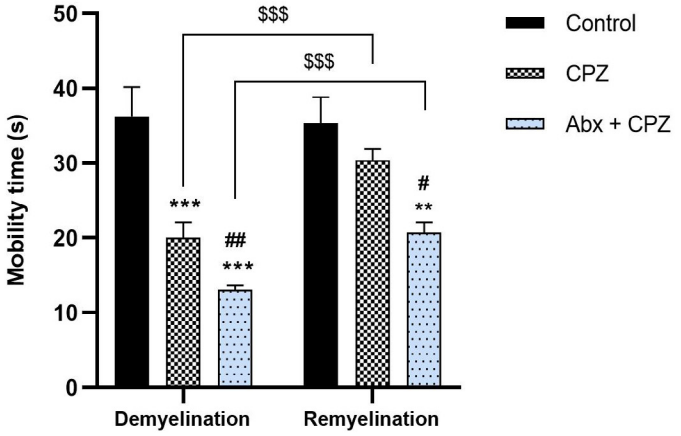

Figure 5: Rotarod Test results quantifying Mobility Time during demyelination and remyelination in the Cuprizone (CPZ)-induced toxic Model. Abx: broad-spectrum antibiotics. The data are presented as mean ± S.E.M. $p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. CPZ treated group; $^{\$}p < 0.05$ for comparisons between the demyelination and remyelination phases.

The results showed a significant reduction in mobility percentage in both the CPZ (20%) and the Abx + CPZ group (19%) compared to controls (37%) during the demyelination phase ($p < 0.001$).

During remyelination, the CPZ group demonstrated a significant improvement in mobility percentage (31%) compared to their performance during demyelination ($p < 0.01$). Despite this progress, their mobility percentage remained significantly lower than controls ($p < 0.05$)

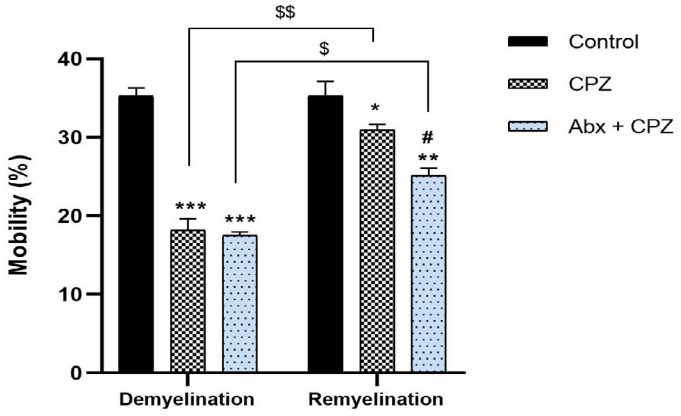

Figure 6: Hanging Wire Test (HWT) results quantifying Mobility Percentage during demyelination and remyelination in the Cuprizone (CPZ)-induced toxic Model. Abx: broad-spectrum antibiotics. The data are presented as mean ± S.E.M. $p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. CPZ treated group; $^{\$}p < 0.05$ for comparisons between the demyelination and remyelination phases.

Similarly, the Abx + CPZ group showed a significant increase in mobility percentage during remyelination

(25%) compared to demyelination ($p < 0.05$). However, their performance remained persistently reduced relative to controls (38%, $p < 0.01$) and was also significantly lower than that of the CPZ group ($p < 0.05$) as shown in Figure 6.

Assessment of oxidative stress in the PFC, HPC, and CB

NO concentrations

As shown in Figure 7, the NO assay results during the demyelination phase reveal that the NO levels are significantly elevated in both the CPZ and Abx + CPZ groups compared to controls ($p < 0.001$) across the prefrontal cortex PFC, hippocampus HPC, and cerebellum CB. Notably, there was a significant difference between the CPZ and Abx + CPZ group in the HPC and CB ($p < 0.01$); however, no such difference was observed in the PFC.

Transitioning from demyelination to the remyelination phase, the NO levels in both the CPZ and Abx + CPZ groups decreased significantly ($p < 0.001$) in the three examined brain structures. Despite this improvement, NO levels in the Abx + CPZ group remain significantly elevated compared to controls during remyelination in the PFC ($p < 0.001$), HPCC ($p < 0.01$), and CB ($p < 0.05$). Additionally, significant differences persist between the Abx + CPZ and CPZ group in the PFC ($p < 0.01$), and HPC ($p < 0.05$).

Lipid peroxidation concentrations

As shown in Figure 8, lipid peroxidation assessed by TBARS levels during the demyelination phase was significantly elevated in both the CPZ and Abx + CPZ groups compared to controls in the PFC ($p < 0.001$) and CB ($p < 0.05$). Notably, in the HPCC, TBARS levels were higher in the Abx + CPZ group compared to controls ($p < 0.001$), whereas the CPZ group showed a less pronounced but still significant increase relative to controls ($p < 0.01$). Furthermore, TBARS levels in the PFC were significantly greater in the Abx + CPZ compared to the CPZ group ($p < 0.01$). In the CB, TBARS levels also differed significantly between the CPZ and the Abx + CPZ ($p < 0.05$). No significant difference between these two groups was observed in the HPC.

Transitioning from demyelination to the remyelination phase, TBARS levels in the CPZ group decreased significantly, particularly in the HPC ($p < 0.001$), CB ($p < 0.01$), and PFC ($p < 0.05$). In the Abx + CPZ group, a significant reduction was observed only in the HPC ($p < 0.05$).

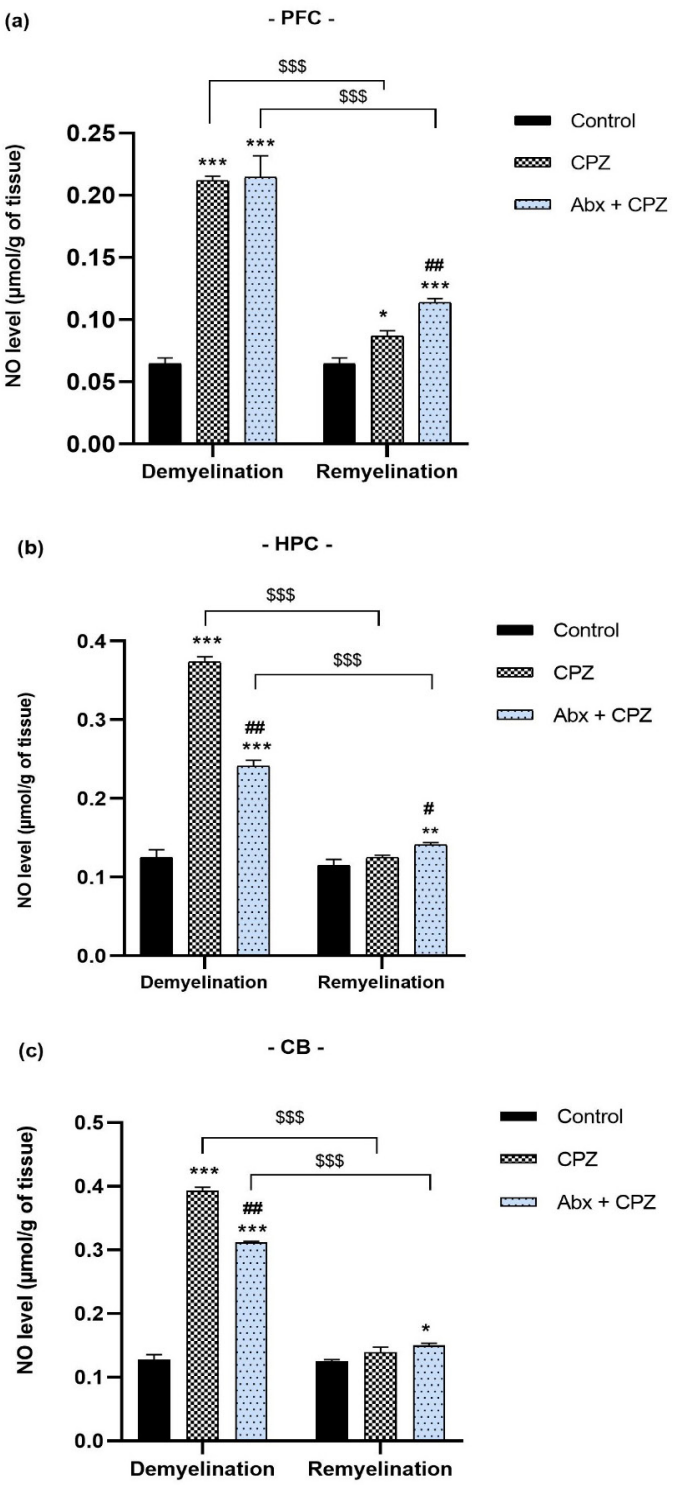

Figure 7: Results of Nitric Oxide (NO) levels quantified in three cerebral structures (a) prefrontal cortex (PFC), (b) hippocampus (HPC), and (c) cerebellum (CB). The data are presented as mean ± S.E.M. $p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. CPZ treated group; $^{\$}p < 0.05$ for comparisons between the demyelination and remyelination phases.

Despite this improvement, TBARS levels in the Abx/CPZ group remained significantly elevated compared to controls during the remyelination phase in the PFC ($p < 0.001$), HPC ($p < 0.01$), and CB ($p < 0.05$). Additionally, significant differences persisted between the Abx + CPZ

and CPZ group in both the PFC and the HPC ($p < 0.01$). For the CPZ group, although TBARS levels improved, they also remained significantly higher than controls in the PFC ($p < 0.01$) and HPC ($p < 0.05$).

Level of catalase activity

As shown in Figure 9, catalase activity during the demyelination phase was significantly decreased in the CPZ group compared to controls across all three examined

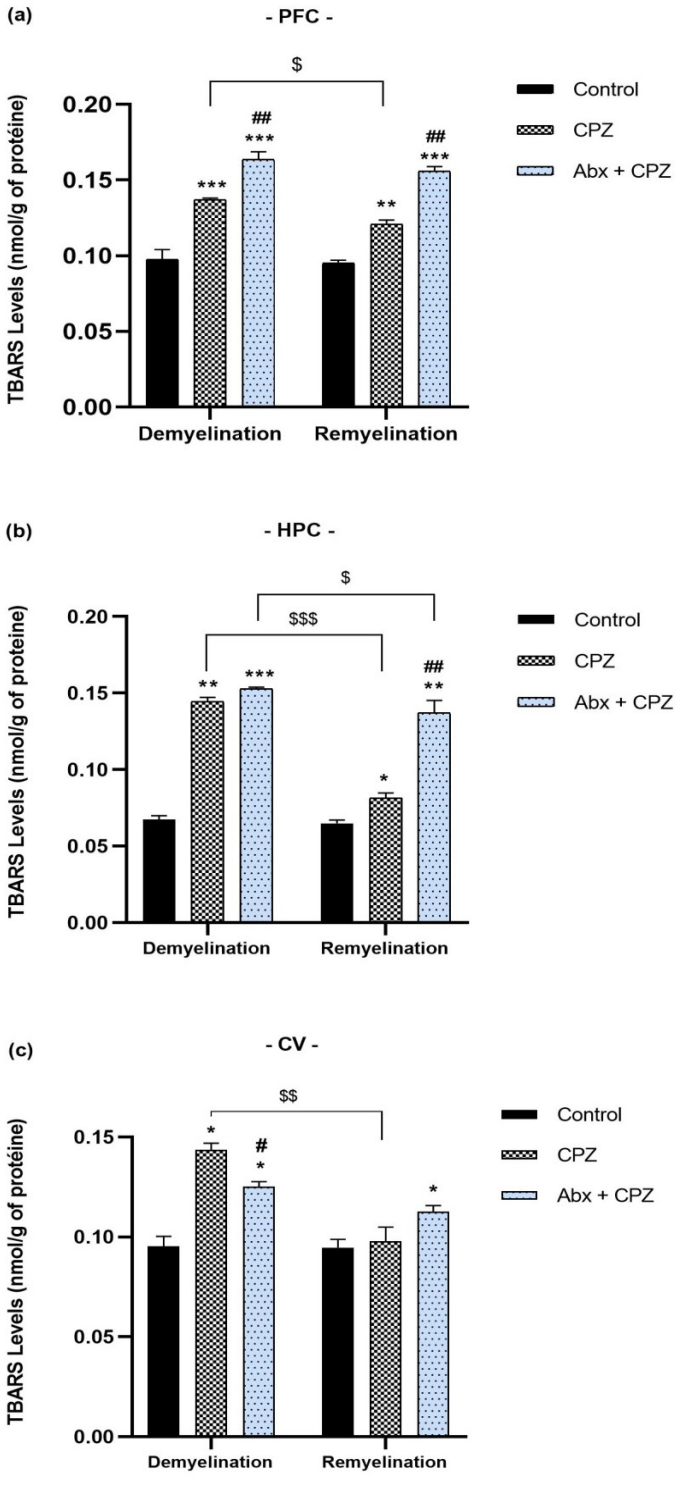

Figure 8: Results of Thiobarbituric Acide Reacting Substances (TBARS) levels measured in three cerebral structures: (a) prefrontal cortex (PFC), (b) hippocampus (HPC), and (c) cerebellum (CB). The data are presented as mean ± S.E.M. $p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. Cuprizone (CPZ) treated group; $^{\$}p < 0.05$ for comparisons between the demyelination and remyelination phases.

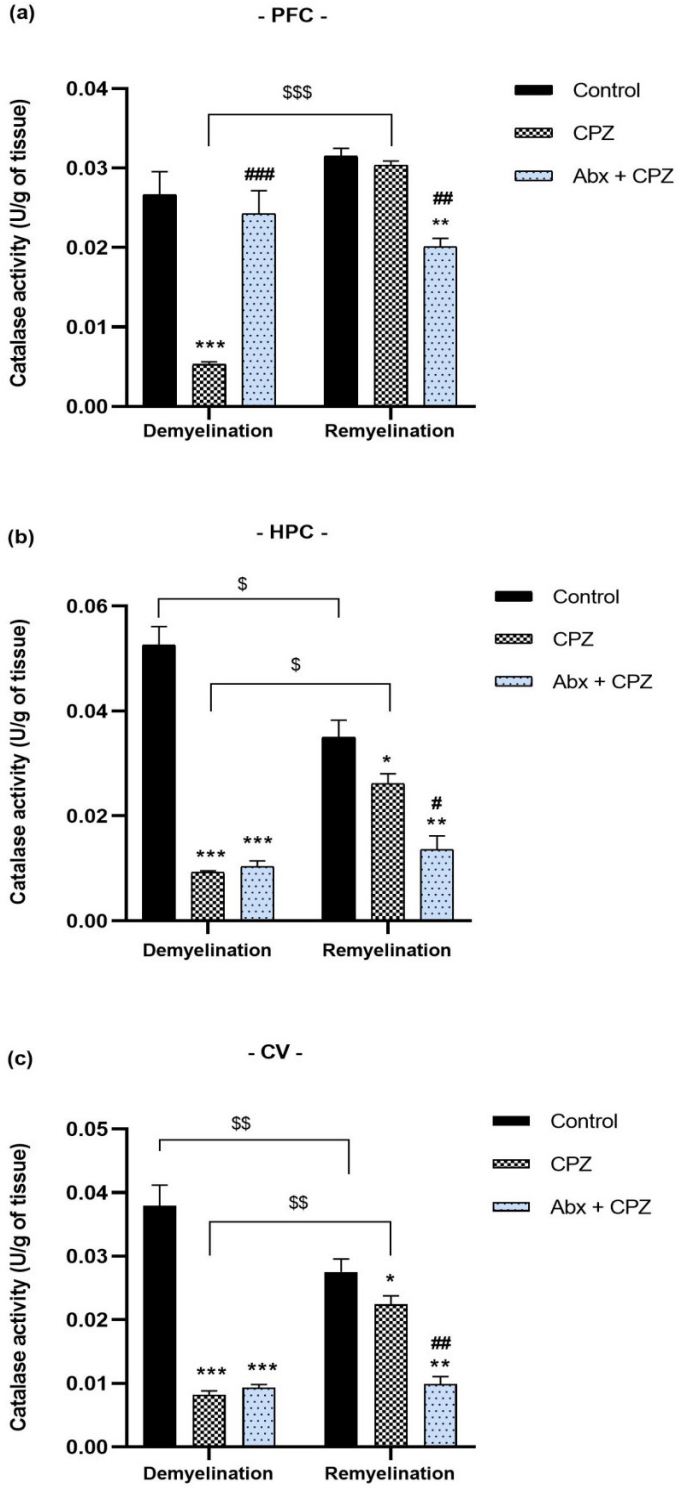

Figure 9: Results of Catalase activity quantified in three cerebral structures: (a) prefrontal cortex (PFC), (b) hippocampus (HPC), and (c) cerebellum (CB). The data are presented as mean ± S.E.M. $p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. Cuprizone (CPZ) treated group; $^{\$}p < 0.05$ for comparisons between the demyelination and remyelination phases.

brain regions the PFC, HPC, and CB with a uniform level of significance ($p < 0.001$). In the remyelination phase, catalase activity increased markedly in the PFC, approaching control levels ($p < 0.001$). Although significant improvements were also noted in the CB ($p < 0.01$) and HPCC ($p < 0.05$), activity in these regions remained significantly lower than controls ($p < 0.05$).

Conversely, during demyelination, the Abx + CPZ group showed no significant difference in catalase activity in the HPC and CB relative to the CPZ group; however, both groups exhibited significantly reduced activity in these regions compared to controls ($p < 0.001$). Interestingly, in the PFC during demyelination, catalase activity in the Abx + CPZ group was comparable to controls and significantly higher than in the CPZ group ($p < 0.01$). In the remyelination phase, catalase activity continued to improve in the CPZ group but paradoxically decreased in the Abx + CPZ group, showing significant reductions compared to both controls and the CPZ group ($p < 0.01$). Within the Abx + CPZ group, catalase activity did not differ significantly between the demyelination and remyelination phases in any of the three brain regions.

Myelination assessment by LFB staining

To examine myelin distribution, coronal brain sections were stained using LFB, with emphasis on the corpus callosum. Representative images were captured at 4× magnification (Figure 10A). In control mice, the CC appeared well-defined and densely myelinated throughout both the demyelination and remyelination phases. In contrast, CPZ -treated mice exhibited prominent white bands indicative of demyelination (as indicated by the red arrows) during the demyelination phase, which largely disappeared during remyelination. Similarly, the Abx + CPZ group displayed demyelinated areas within the CC during demyelination. Although these areas were reduced after the remyelination phase, reduction in demyelination persisted to a noticeable extent (as shown by the red arrow).

Statistical analysis of myelin integrity using ImageJ image processing revealed highly significant demyelination in both the CPZ (30%) and Abx/CPZ (35%) groups compared to controls (13%) during the demyelination phase ($p < 0.001$, Figure 10B). Notably, demyelination was significantly more severe in the Abx + CPZ compared to the CPZ group ($p < 0.05$). During the remyelination phase, the CPZ group (14%) showed a clear improvement in myelin restoration ($p < 0.001$); however, the myelination percentage remained significantly lower than controls (11%, $p < 0.05$). Although the Abx + CPZ group (20%) also exhibited some improvement, the level of demyelination remained significantly greater compared to both the control ($p < 0.001$) and CPZ ($p < 0.01$) groups. These results indicate that neonatal gut microbiota depletion via antibiotics exacerbated demyelination and impaired remyelination efficiency in the CPZ model.

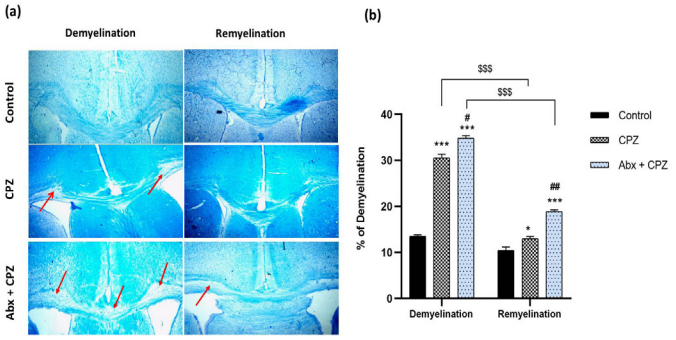

Figure 10: Luxol Fast Blue (LFB) staining of the corpus callosum (a) and quantification of demyelination (b). The data are presented as mean ± S.E.M. $p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. Cuprizone (CPZ) treated group; $^{\$}p < 0.05$ for comparisons between the demyelination and remyelination phases.

DISCUSSION

Our results demonstrate that neonatal depletion of gut microbiota with a cocktail of broad-spectrum absorbable antibiotics (amoxicillin, clarithromycin, and metronidazole) exacerbates behavioral, oxidative, and neuropathological impairments in the CPZ model of demyelination and remyelination in Swiss albino mice. These findings reinforce the emerging concept of the gut-brain axis as a key modulator of neurodevelopment and neuroprotection, but also highlight important caveats regarding the interpretation of antibiotic effects

Consistent with previous studies, our data show that early-life microbiota depletion leads to persistent anxiety- and depression-like behaviors, cognitive deficits, and impaired motor performance (Cryan *et al.*, 2019; Morais *et al.*, 2021). The antibiotic-treated group exhibited more severe and less reversible deficits than the CPZ group, supporting the notion that gut microbiota plays a crucial role in shaping neurodevelopmental trajectories and modulating resilience to demyelinating insults (Hoban *et al.*, 2017; Hayer *et al.*, 2023).

Biochemically our results reveal that neonatal gut microbiota depletion via broad-spectrum, absorbable antibiotics leads to a pronounced and persistent imbalance in oxidative stress markers in the adult brain following cup-induced demyelination. This is evident across all three brain regions analyzed the PFC, HPC, and CB and is characterized by elevated NO and lipid peroxidation (TBARS), alongside reduced catalase activity. The significant elevation of NO levels during the demyelination phase in both the CPZ

and antibiotic-treated groups, with even higher levels in the antibiotic group (notably in HPC and CB), strongly suggests that neonatal gut microbial disruption amplifies neuroinflammatory responses. NO is a reactive nitrogen species produced by activated microglia and astrocytes during neuroinflammation and is known to contribute to oligodendrocyte injury and myelin loss (Wang and Kasper, 2014; Braniste *et al*., 2014). The persistent elevation of NO in the antibiotic group during remyelination, despite partial recovery, indicates a sustained pro-oxidant environment that may hinder effective myelin repair. Similarly, TBARS levels reflecting lipid peroxidation were significantly higher in the antibiotic group across most brain regions, particularly in the PFC and HPC. Suggesting that neonatal microbiota depletion not only exacerbates initial oxidative damage during demyelination but also impairs the resolution of oxidative stress during remyelination.

Catalase, a key antioxidant enzyme, was markedly decreased in both experimental groups during demyelination, but the antibiotic group showed a distinct pattern: while catalase activity in the PFC was initially preserved, it failed to recover during remyelination and even declined further, remaining significantly lower than both controls and the CPZ group. This paradoxical suppression of catalase activity after neonatal microbiota depletion suggests that the early-life microbial environment is crucial for establishing robust, long-lasting antioxidant defenses in the brain (Hyży *et al*., 2025). The inability to restore catalase activity may underlie the persistent oxidative stress and impaired remyelination observed in the antibiotic group.

Histologically, the antibiotic-treated group had more severe demyelination and less efficient remyelination in the corpus callosum compared to controls and the CPZ group. Although some remyelination occurred after CPZ withdrawal, myelin restoration remained incomplete in animals with early-life microbial disruption. These findings suggest that neonatal gut microbiota depletion increases vulnerability to demyelinating injury and hampers recovery, likely through persistent neuroinflammation and impaired oligodendrocyte function, as supported by many studies (Hoban *et al*., 2016; Rothhammer *et al*., 2018; Zhao *et al*., 2024).

While our results strongly implicate the gut microbiota in modulating these outcomes, it is important to acknowledge that the antibiotics used in this study amoxicillin, clarithromycin, and metronidazole are well absorbed and can exert systemic effects beyond the gastrointestinal tract (Leclercq *et al*., 2017). These agents can cross the blood-brain barrier to varying degrees, and potentially influence CNS oxidative pathways independently of microbiota depletion. However, the persistent and region-specific oxidative stress observed here, particularly during remyelination, is consistent with a loss of microbiota-derived neuroprotection rather than a transient pharmacological effect.

Limitations

This study could present some inherent limitations related to the use of antibiotics and the characteristics of the CPZ model, which should be taken into account when interpreting the findings. To bridge these gaps, further studies are required to reveal the underlying pathways connecting early gut microbiota disruption with oxidative stress and myelin damage. Additionally, investigating microbiota-targeted therapies could offer promising avenues to reduce the neurological deficits observed.

CONCLUSIONS AND RECOMMENDATIONS

In summary, our study demonstrates that neonatal disruption of gut microbiota increases susceptibility to myelin damage, impairs repair processes, and worsens neurobehavioral outcomes in adulthood within a non-inflammatory CPZ model. Early-life antibiotic-induced microbiota depletion resulted in heightened oxidative stress and altered antioxidant defenses, in critical brain regions involved in cognition, motor control, and mood regulation. This work supports and extends previous findings on the gut-brain axis by demonstrating that gut microbiota integrity during early development is essential for maintaining myelin homeostasis and neuroprotection in adulthood, even in non-inflammatory demyelination models such as CPZ. By highlighting the lasting impact of neonatal gut dysbiosis on myelin integrity and antioxidant mechanisms, our findings broaden the understanding of the gut-brain axis beyond inflammatory contexts and suggest that preserving microbiota integrity during development may be crucial for lifelong neurological health.

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NOVELTY STATEMENT

This study significantly advances understanding of how early-life gut microbiota disruption impacts non-inflammatory demyelination, addressing a critical gap left by the predominant focus on experimental autoimmune encephalomyelitis models. By specifically targeting the neonatal period, a crucial window for neurodevelopment, our findings demonstrate that early microbiota disturbances exacerbate myelin damage, increase oxidative stress, and

lead to lasting neurobehavioral impairments in adulthood. Ultimately, this work establishes a novel link between neonatal microbiota depletion and functional cognitive and motor deficits in a non-inflammatory demyelination model, thereby expanding current knowledge on the long-term neurological consequences of early microbial insults.

AUTHOR'S CONTRIBUTION

AD and ZE-M: Conceptualization, methodology, software, data curation, visualization, investigation, validation, writing, and editing.
ML and LI-D: Conceptualization, data curation, visualization, writing, reviewing, and editing.
HM, HL and KC: Conceptualization, writing, reviewing, and English editing.
TT: Conceptualization, methodology, software, visualization, investigation.
A E-H: Conceptualization, methodology, software, writing, reviewing, and editing.
AM: Conceptualization, methodology, software, data curation, visualization, investigation, supervision, validation, writing, reviewing, and editing.

Abbreviation

ABX: Broad spectrum antibiotics; CAT: Catalase; CB: Cerebellum; CC: Corpus callosum; CNS: Central nervous system; CPZ: Cuprizone; HPC: Hippocampus; HWT: Hanging wire test; IACUC: International Animal Care and Use Committee; LFB: Luxol Fast Blue; LPO: Lipid peroxidation; MS: Multiple sclerosis; NIH: National Institutes of Health; NO: Nitric oxide; NOR: Novel Object Recognition; OFT: Open field test; PFA: paraformaldehyde; PFC: Prefrontal cortex; PND: Postnatal day; SEM: Standard error of the mean; TBARS: Thiobarbituric-acid-reacting substances; TST: Tail Suspension Test.

Generative AI and AI-assisted technology statement

The authors acknowledge that generative AI and AI-as sisted technologies (such as Grammarly and ChatGPT) were used only to improve English grammar and mini mize mistakes in this manuscript to ensure better quality. No AI technologies were used to generate, analyze, or in terpret scientific data. The authors take full responsibility for the content and conclusions of this final manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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