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Protective Effect of Probiotic *Enterococcus faecium* NCIM 5593 on Acrylamide Induced Neurotoxicity in Adult Mice

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Abstract

Exposure to chemicals that are commonly distributed in the environment and work-related surroundings may have deleterious effects to the nervous system. Acrylamide (ACR) is a well-known neurotoxin with multiple chemical and industrial applications. ACR exposure is attributed to oxidative stress and is known to cause neurotoxic effect by altering brain neurotransmitter levels. Probiotics are chosen as natural therapeutic medicine against oxidative stress and shown their ability to modulate gut-brain axis. Aim of the present study was to evaluate the beneficial effect of probiotic *Enterococcus faecium* NCIM 5593 on ACR induced oxidative stress altered neurotransmitter status in mice brain. ACR exposure to mice produced pronounced neurotoxicity as evidenced by marked increase in oxidative markers and altered antioxidant ability. Probiotic treatment (4 weeks) to young mice could diminish ACR induced elevation in oxidative markers in brain and enhance activities of antioxidant enzymes with increase in gamma-aminobutyric acid (GABA) and dopamine (DA) levels. Oral supplements of *E. faecium* NCIM 5593 to ACR-treated mice improved neuronal dysfunction and oxidative stress. The present study suggests that this probiotic strain can be a potential neutraceutical intervention to combat acrylamide induced molecular alterations and oxidative stress.

Keywords: Probiotic; Acrylamide; Oxidative stress; Oxidative markers; Antioxidant enzymes; Neutraceutical intervention

Introduction

Accumulating evidence suggests the interaction between gut microbiota and central nervous system (CNS) is through microbiome-gut-brain axis. Earlier studies support the role of probiotics to positively influence indigenous microflora [1], inhibit carcinogenesis [2], induce non-specific activation of the host's immune system [2] and exhibit anti-hyperlipidemic [3,4], and anti-colitic effects [5]. Furthermore, probiotics have also shown their profound ability to impact brain function [6]. Probiotic formulation consisting of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175A administered to human volunteers and rat models significantly attenuated psychological distress and also reduced anxiety-like behavior [7]. Supplements of *Bifidobacterium infantis* to rats resulted in neurochemical alterations [8]. In line with this, our previous study demonstrated that *E. faecium* NCIM 5593 supplements could modulate behavioural phenotypes and endogenous antioxidant defences in young mice brain [9]. However, their protective effect against neurotoxicant induced oxidative stress has not been studied.

Acrylamide [ACR, C_3H_5NO; prop-2-enamide] is a well-recognized neurotoxin which is widely used in waste water treatment, textile industry and ore processing. Its wide spread application is associated with pollution and health risks [10]. It is also a common contaminant in foods prepared by cooking at high temperatures [11]. Even though, occupational ACR exposure in humans is recognized as the primary risk factor, there is a growing concern about the potential health effects of low sub-chronic ACR exposure through commonly consumed thermally processed foods [12]. The importance of ACR in food was showed by Tareke et al. [13] by feeding rats with fried feed which led to a large increase in the levels of haemoglobin adduct, which was concluded to be N-(2-carbamoyl methyl) valine. In line, Swedish researchers reported that heat treated starch rich foods such as potato and cereal products contained high levels of ACR [13,14]. It is well established that the asparagine, a free amino acids and reducing sugars in cereals and potato, are crucial participants in the production of ACR by Strecker degradation mechanism of Maillard reaction [15]. In addition, processing conditions, such as temperature, water activity and matrix, influence its formation [16]. Even though it is reported that a temperature of 120°C or higher is needed for the ACR formation, there are reports authenticating that ACR formation occurs even at temperatures below 100°C, particularly in drying processes at 65-130°C [17].

The neurotoxicity of ACR has been extensively studied with respect to mammalian species (i.e., rats, mice, monkeys, guinea pigs, dogs, cats) and with daily dose-rates of 0.5-50 mg/kg per day [18-20]. Furthermore, it has been reported that ACR induced neurotoxicity induces reactive oxygen species (ROS) formation, lipid peroxidation and reduces antioxidant capacity in CNS. Therefore, oxidative stress plays important role in ACR induced toxicity.

In our present investigation, we sought to access whether oral supplements of *E. faecium* NCIM 5593 would ameliorate ACR induced oxidative stress and neurotoxicity.

Experimental Methods

Chemicals

de Man Rogosa and Sharpe (MRS) broth was obtained from Hi Media Laboratories, India. All other chemicals used were of analytical grade and were procured from Sigma-Aldrich, USA.

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Bacterial strains and growth conditions

Bacterial strain, *E. faecium* NCIM 5593 used in the study was isolated from indigenous fermented food and identified using genomic techniques, *viz.,* 16 rRNA and ITS method [21]. *E. faecium* NCIM 5593 has earlier been reported to be a potent GABA producer *in vitro* and under simulated gastro-intestinal conditions [22,23]. *Lactobacillus rhamnosus* GG MTCC 1408 was used as a positive control in the study. The strains were maintained at -80°C in de Man Rogosa and Sharpe (MRS) broth with 10% (v/v) glycerol and were normally cultured in MRS broth at 37°C under static conditions.

Animals and care

Male mice (CFT-Swiss; 6 weeks old; 20-25 grams) were obtained from CSIR-Central Food Technological Research Institute (CSIR-CFTRI) animal facility. The animals were housed in rectangular polypropylene cages (27 × 21 × 14 cm; three per cage) provided with dust free paddy husk as bedding material. Cages were maintained under standard experimental conditions: photoperiod 12-h light/dark cycle; RT 25 ± 2°C, humidity 40-60%. Mice were acclimatized for one week prior to the start of the experiment and were maintained on commercial diet and tap water *ad libitum*. All experimental test paradigms were approved by the Institutional Animal Ethics Committee and were conducted in adherence with the guidelines stipulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Ministry of Environment, Forests and Climate Change, Government of India, India.

Group assignment and dose regime

After one week of acclimation, the mice were assigned at random to one of the following treatment groups (n=6): control (CTR) group mice received saline p.o.; Lb and EF group mice received *L. rhamnosus* GG MTCC 1408 and *E. faecium* NCIM 5593 [10^8 CFU/d, p.o.] respectively; A5 and A10 group mice received ACR at 5 and 10 mg/kg BW, respectively (*i.p.*, thrice a week); A5+Lb and A5+EF group mice received ACR 5 mg/kg BW (*i.p.*, thrice a week) and *L. rhamnosus* GG MTCC 1408 or *E. faecium* NCIM 5593 [10^8 CFU/d]; A10+Lb and A10+EF group mice received ACR 10 mg/kg BW (*i.p.*, thrice a week) and *L. rhamnosus* GG MTCC 1408 or *E. faecium* NCIM 5593 [10^8 CFU/d]. At 28th day, all mice were tested for affective-like behaviours: open field test and elevated plus maze between 09:00 and 14:00 hours. Finally, mice of all groups were scarified 2 h following the final treatment, brain was excised and brain regions *viz*., cortex (Ct), cerebellum (Cb) and hippocampus (Hc) were dissected and stored at -80°C until further processing. Cytosolic fractions were prepared from each region and biochemical estimations and neurochemical quantification was carried out.

Behavioral assessment

Open field test and elevated plus maze: Open Field Test: Mice were placed individually in a corner of the arena (illuminated by ambient lights) of an open filed box and allowed to freely explore the open field for 10 min by videotaping. Indices of exploratory movement including the number of entries into and total duration of time spent in the center zone were recorded. The test box was cleaned with 70% ethanol after videotaping each mice movement.

Elevated Plus Maze: Mice were individually placed at the junction of the open and closed arms of elevated plus maze unit (consisted of four arms; two open without walls and two enclosed by 16 cm high walls; the unit is 30 cm long and 5 cm wide. The arms were connected with a central square of dimensions 5 × 5 cm and each arm of the maze was attached to sturdy wooden legs such that it is elevated 40 cm off perpendicular to the floor), facing the open arm and permitted free exploration for 10 min. The mice behavior was videotaped for 10 min, during which their entries onto open and closed arms and the time spent in each arm were recorded following a four-paw criterion. Each arm was cleaned with 70% ethanol between each trails.

Brain dissection

Brain regions *viz.,* Ct, Cu and Hc were dissected from both hemispheres. Standardized protocol was followed for the isolation of cytosolic fractions [9]. Briefly, 10% (w/v) homogenate were prepared in ice-cold isolation buffer (100 mM sucrose, 10 mM EDTA, 100 mM Tris-HCl; pH 7.4) using a glass-Teflon grinder. Homogenate was then centrifuged at 1000 × g for 10 min at 4°C to remove nuclei. Subsequently, cytosolic fractions isolated by differential centrifugation at 10,000 × g for 20 min at 4°C was stored at -80°C until further use.

Oxidative stress markers

Reactive oxygen species (ROS): ROS levels were assayed using 2',7'-dichlorodihydrofluorescein (DCFH-DA) assay. DCFH-DA is deacetylated to 2', 7'-dichlorofluorescein (DCFH) and subsequently it gets oxidized to a fluorescent compound, dichlorofluorescein (DCF) by ROS

(Gokul and Muralidhara, 2015). Briefly, an aliquot (~100 ug protein) was incubated with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 5 mM HEPES, 2 mM CaCl2 and 10 mM glucose; pH 7.4) and 10 ul DCFH-DA (5 uM) was incubated at RT. After 30 min of incubation, the fluorescent compound (DCF) is detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 and 530 nm, respectively. ROS formation was quantified from a standard DCF-standard curve and the result is expressed as pmol DCF formed/min/mg protein [9].

Lipid peroxidation (LP)

The extent of LP was assessed by measuring free malondialdehyde levels (MDA). Briefly, an aliquot (~ 500 ug protein) was made to react with 1.5 ml of acetic acid (2.5 M HCl, pH 3.5) and 1.5 ml of thiobarbituric acid (0.8% w/v) at 95°C for 45 min. After cooling to RT, the pink chromogen formed [(thiobarbituric acid) 2-MDA] was measured at 532 nm quantified as MDA equivalents using 1,1,3,3-tetramethoxypropane as the standard [24].

Nitric oxide (NO)

NO levels were measured using commercially available Griess reagent. The assay principle is based on enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is based on two step diazotization reaction in which acidified NO2 produces a nitrosating agent which reacts with sulphanilic acid to produce the diazonium ion which then couples with N-1-napthylethylenediamine to form the chromophoric azo derivative which absorbs light at 540 nm. NO levels were quantified from a sodium nitrate standard curve [25].

Protein carbonyls (PC)

Levels of PC were determined as described previously [9]. In brief, an aliquot (~500 ug protein) was added to TCA (2%, 200 µl) and centrifuged at 10,000 × g for 10 min at 4°C. The resulting pellet was suspended in 1 ml 2,4-dinitrophenyl hydrazine (10 mM in 2 N HCl) and kept at dark for 1 h. TCA (20%, 500 µl) was added to precipitate the protein and the pellet was washed in acetone (1 ml) and dissolved

in SDS (2%, 1 ml) prepared in 20 mM Tris-HCl. The absorbance was recorded at 360 nm and the results were expressed as nmoles carbonyls/mg protein (ε-22,000/M/cm).

Reduced glutathione (GSH)

GSH levels were quantified by fluorescence detection after reaction of sample aliquots containing formic acid/NaH_2PO_4/EDTA with O-phthalaldehyde (OPT). In brief, cytosol, (0.1 mg protein) suspended in 1.5 ml phosphate buffer (0.1 M NaH_2PO_4, 5 mM EDTA; pH 8.0) and 500 µl formic acid (0.1 M) was centrifuged at 10,000 × g for 20 min. Supernatant (100 µl)was added to phosphate buffer (1.8 ml) and OPT (100 µl, 1 mg/ml). After thorough mixing, fluorescence intensity was measured at activation/emission wavelengths of 345/425 nm. Concentration of GSH was calculated from the standard curve and the results are expressed as µg GSH/mg protein [9].

Enzyme activity assays

Catalase (CAT) and superoxide dismutase (SOD) activities: CAT activity was determined by the method described previously [9] and results are expressed as nmol H_2O_2 consumed/min/mg protein (ε-43.6/mM/cm). SOD activity was assayed by monitoring the inhibition of quercetin auto-oxidation [9].

Acetylcholine esterase (AChE) activity: AChE activity was determined as described previously [9]. In brief, to the reaction mixture containing 1 ml phosphate buffer (0.1 M, pH 8.0), 50 µl DTNB (10 mM), cytosolic sample (0.01 mg protein) and 20 µl acetylcholine iodide (78 mM) were added and change in the absorbance was monitored over 3 min at 412 nm. Enzyme activity is expressed as nmole substrate hydrolyzed/min/mg protein.

HPLC determination of monoamine levels

GABA levels: GABA in the cytosolic fractions were analyzed by derivatizing sample with O-phthalaldehyde-mercaptoethanol reagent [OPA-MCE (20 µl), prepared by dissolving 10 mg OPA and 10 µl MCE in 2.5 ml acetonitrile] and boric acid buffer (100 µl, 0.4 M; pH 10.4) as previously described [22]. Derivatization was carried out at 30 ± 2°C for 5 min, and the reaction mixture was filtered through microfilters (pore size 45 lm; Millipore, Bedford, MA). The analysis was carried out using by passing the samples through a LC-18 analytical column (150 mm × 4.6 mm, 5 µm particle size) connected to a UV detector (338 nm) at a flow rate of 0.8 mL/min. Mobile phase (1l) consisted of sodium acetate (1.64 g), triethylamine (200 µl) and acetonitrile (20% v/v). GABA levels were calculated from an external standard and results are expressed as µg GABA/mg protein.

DA levels: The samples (20 µl) were passed through LC-18 analytical column (150 mm × 4.6 mm, 5 µm) connected to a UV detector (280 nm). Mobile phase consisted of trifluoroacetic acid (0.2% v/v) and methanol (70:30 v/v; pH 3.5) in deionized water. The mobile phase was subsequently filtered and degassed, following which pH was adjusted to 3.5. The flow rate of 1 ml/min was maintained. DA levels in the sample were calculated from external standard and expressed as µg DA/mg protein [26].

Statistical analysis

The experimental data obtained were expressed as mean + SE. The differences were analyzed by one-way ANOVA followed by a post-hoc Turkey's test. Statistical analysis was performed using InStat 3 software (v3.36).

Results

Body weight

Oral supplementation of *E. faecium* NCIM 5593 and *L. rhamnosus* GG MTCC 1408 in growing mice had no significant effect on body weight gain. ACR administration caused marked reduction in mice body weight, while those given probiotic supplements along with ACR showed moderate improvement (Table 1). Further, mice provided with probiotic supplement showed no signs of behavioural abnormalities (in terms of gait/posture and physical observations of any generalized systemic toxicity) during the experimental period.

Behavioral assessment

In the open field test, two fundamental indices of exploratory measures *viz.,* the number of entries into the center zone and the total time spent in the center of the open field arena were analyzed (Figures 1a and 1b). Supplementation of probiotics to normal mice didn't significantly increase the frequency of entries to the centre arena. Mice exposed to ACR exhibited marked reduction in the number of entries in the center zone, while ACR mice provided probiotic supplements enter the center zone of the arena more frequently than did their ACR alone counterparts (Figure 1a). Likewise, in terms of time spent exploring the center of the open field, mice supplemented with probiotic supplements *per se* showed significant increase in time spent. ACR mice spent significantly less time exploring the center, while those receiving probiotic supplements spent relatively more time in the central part of the arena (Figure 1b). Likewise, in elevated plus maze, supplementation of probiotics significantly increased the number of entries to open arm (Figure 2a). Conversely, number of entries were reduced in ACR treated mice. Interestingly, probiotic supplements

Treatment group	Body weight (g)				
	Initial	Week 1	Week 2	Week 3	Week 4
CTR	29.65 ± 0.12	31.78 ± 0.50	35.09 ± 0.25	37.03 ± 0.43	39.18 ± 0.24
LB	30.12 ± 0.18	32.09 ± 0.24	35.15 ± 0.12	37.15 ± 0.31	38.75 ± 0.46
EF	30.69 ± 0.21	32.98 ± 0.26	35.19 ± 0.28	36.95 ± 0.29	38.26 ± 0.55
A5	30.51 ± 0.13	32.54 ± 0.46	33.36 ± 0.23$^{\#}$	32.41 ± 0.51$^{\#\#\#}$	31.56 ± 0.38$^{\#\#\#}$
A5+LB	31.06 ± 0.15	33.27 ± 0.52	34.14 ± 0.27	34.65 ± 0.53**	35.68 ± 0.49***
A5+EF	30.95 ± 0.19	32.38 ± 0.17	34.14 ± 0.31	35.14 ± 0.29***	35.11 ± 0.27***
A10	29.94 ± 0.22	32.24 ± 0.25	31.12 ± 0.42$^{\#\#\#}$	30.53 ± 0.31$^{\#\#\#}$	30.23 ± 0.22$^{\#\#\#}$
A10+LB	30.51 ± 0.21	33.12 ± 0.23	34.17 ± 0.31$^{\wedge\wedge\wedge}$	34.68 ± 0.25$^{\wedge\wedge\wedge}$	35.77 ± 0.53$^{\wedge\wedge\wedge}$
A10+EF	30.18 ± 0.17	32.83 ± 0.45	34.64 ± 0.62$^{\wedge\wedge\wedge}$	35.09 ± 0.59$^{\wedge\wedge\wedge}$	36.11 ± 0.61$^{\wedge\wedge\wedge}$

Values are mean ± SE (n=6). Data analyzed by one way analysis of variance (ANOVA) followed by post hoc Tukey's test ($^{\#,*,\wedge}$p<0.05; $^{\#\#,**,\wedge\wedge}$p<0.01 and $^{\#\#\#,***,\wedge\wedge\wedge}$p<0.001). Significances were determined by making comparisons between $^{\#}$CTR versus LB, EF, A5 and A10; *A5 versus A5+LB and A5+EF; $^{\wedge}$A10 versus A10+LB and A10+EF

Table 1: Effect of probiotic supplementation on mice body weight under the conditions of ACR induced oxidative stress and neurotoxicity.

could increase the number of entries to open arm in ACR treated mice, however, this effect was not significant. Probiotic supplementation *per se* caused non-significant increase in time spent in open arm (Figure 2b). Time spent in open arm by ACR treated mice were reduced and supplementation of probiotics didn't significantly increase the time spent in open arm.

Oxidative stress markers

Mice fed with *L. rhamnosus* MTCC 1408 and *E. faecium* supplements *per se* showed significant decrease in ROS levels in Hc (34.83%; p<0.05) and Ct (32.53%; p<0.05) regions, respectively. A5 administered mice showed elevated increase in ROS levels in Cu (49.20%; p<0.01) and Hc (35.35%; p<0.05) regions. However, no significant increase in ROS level was observed in Ct region in A5 administered mice. Alternatively, exposure of mice to A10 significantly increased ROS levels in all brain regions (Ct: 35.82%, p<0.05; Cb: 64.84%, p<0.001; Hc: 73.31%, p<0.001). Co-exposed (A5+Lb) mice normalized ROS levels compared to ACR exposed mice in Cb (25.95%, p<0.05) and Hc (40.54%, p<0.01) regions. Significant reduction in ROS level was observed in Hc region in A10+Lb (51.09%, p<0.01) and A10+EF (65.86%, p<0.001) administered mice (Figure 3).

L. rhamnosus MTCC 1408 and *E. faecium* supplements *per se* could effectively reduce MDA levels only in Ct (Lb: 39.82%, p<0.05; EF: 47.61%, p<0.01). ACR exposure resulted in a concentration dependent increase of MDA levels in Ct (A5: 56.25%, p<0.001; A10: 59.61%, p<0.001), Cb (A5: 44.18%, p<0.01; A10: 51.20%, p<0.001) and Hc (A10: 28.54%, p<0.05) regions. No significant increase in MDA level was observed between CTR and ACR exposed mice in Hc region. The elevated levels of MDA induced by A5 (Lb: 25.12%; EF: 22.91%) and A10 (Lb: 21.15%; EF: 28.84%) were significantly (p<0.05) attenuated by both *L. rhamnosus* MTCC 1408 and *E. faecium* supplements in Ct region. Significant decrease (28.57%, p<0.05) in MDA level was observed in ACR10+EF mice in Cb region (Figure 4).

No significant change in NO level was observed in mice supplemented with *L. rhamnosus* MTCC 1408 *per se* in all brain regions (Figure 5). Reduction was observed in Cb (18.88%; p<0.05) and Hc (31.16, p<0.001%) regions of mice supplemented with *E. faecium*. ACR exposure caused significant elevation in NO levels in all brain regions (A5: Ct, 23.45%; Cb, 17.26%; Hc, 17.70% (p<0.05); A10: Ct, 76.90%, p<0.001; Cb, 32.56%, p<0.01; Hc, 31.77%, p<0.01). Increase in NO level evident among ACR exposed mice were significantly restored with supplementation of *E. faecium* NCIM 5593 in Ct (A10+EF: 18.86%, p<0.05), Cb (A5+EF: 17.57%, p<0.05) and Hc (A5+EF: 15.12%, p<0.05; A10+EF: 12.65%, p<0.05) regions. Likewise, *L. rhamnosus* MTCC 1408 supplements could reduce NO levels in ACR exposed mice only in Hc (A5+LB: 15.06%, p<0.05) region.

E. faecium NCIM 5593 supplements *per se* caused a marginal decrease in PC levels in all brain regions, while *L. rhamnosus* GG MTCC

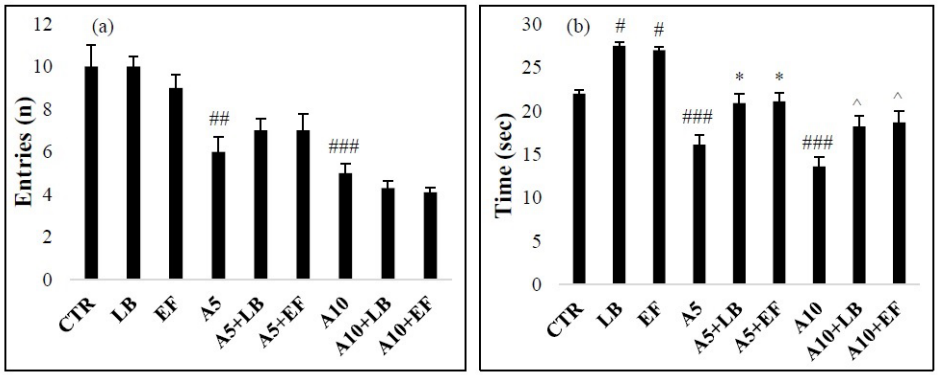

Figure 1: Effect of probiotic supplementation on mice exploratory behaviour in open field test. (a) number of entries into the center zone (b) time spent (sec) in the center zone of the open field box.

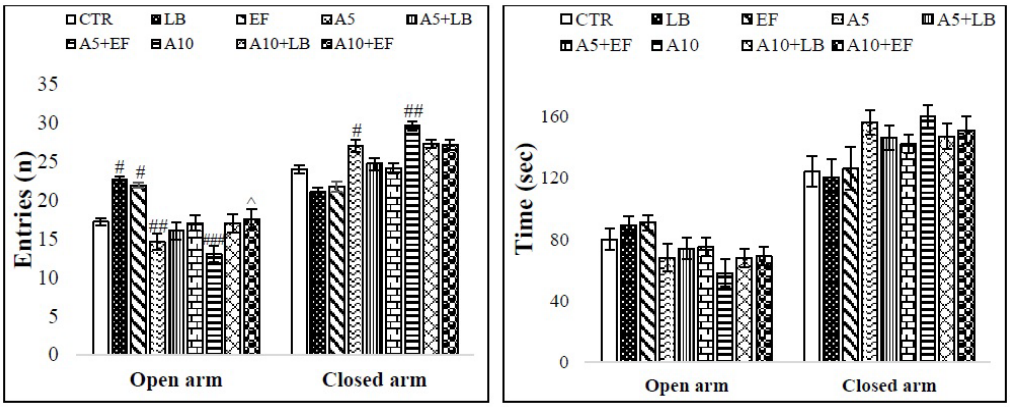

Figure 2: Effect of probiotic supplementation on mice behaviour in elevated plus maze. (a) number of entries to open and closed arm. (b) time spent (sec) in open and closed arm.

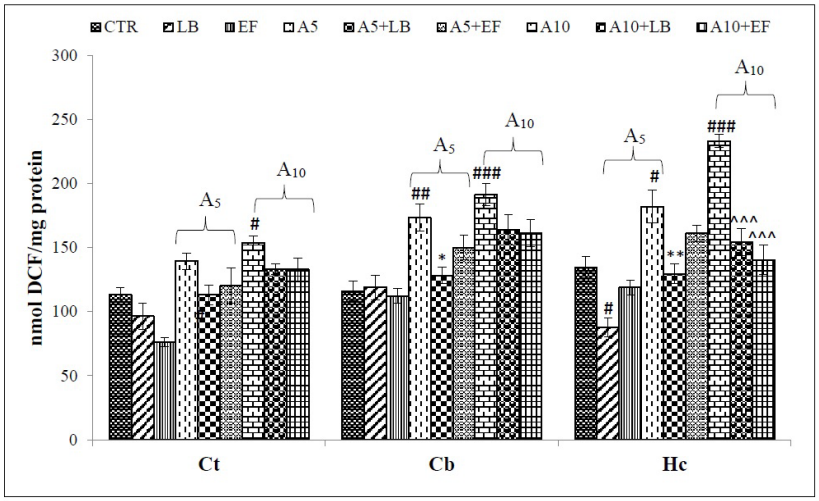

Figure 3: Effect of probiotic supplementation on ROS levels.

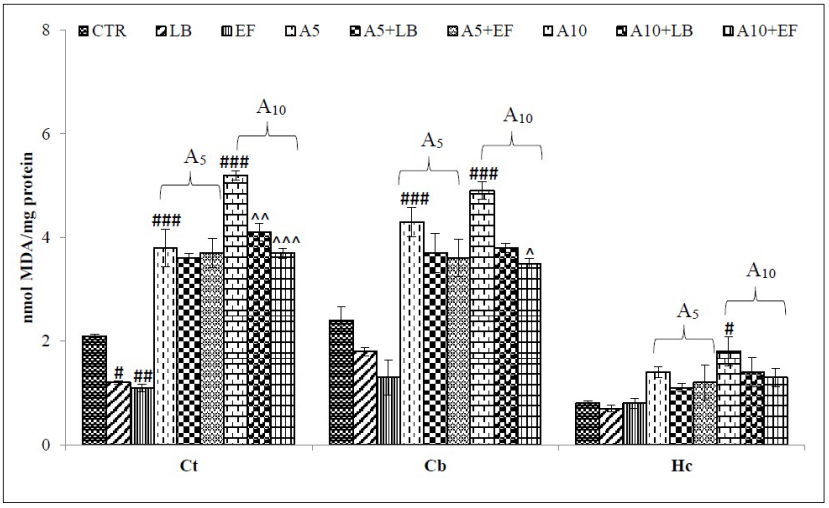

Figure 4: Effect of probiotic supplementation on MDA levels.

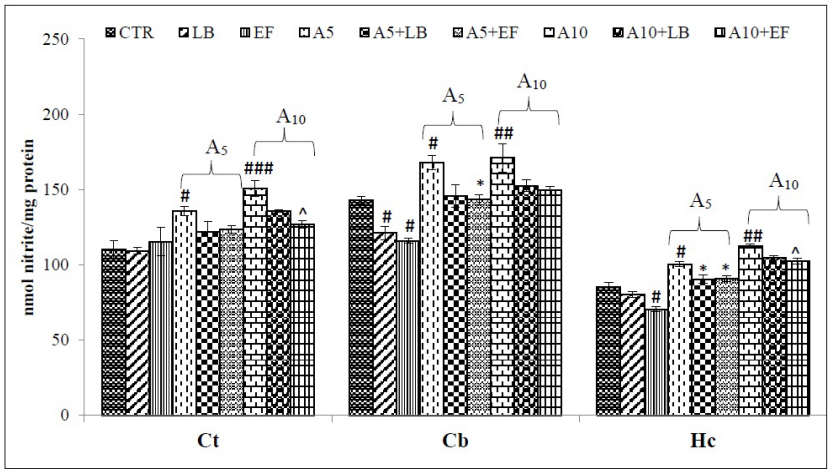

Figure 5: Effect of probiotic supplementation on NO levels.

1408 supplements could effectively reduce PC levels in Ct (22.78%, p<0.05). ACR administration caused significant elevation in PC levels in Ct (A5: 69.74%, p<0.001; A10: 80.10%, p<0.001), Cb (A10: 25.25%, p<0.05) and Hc (A5: 50.09, p<0.01; A10: 78.76%, p<0.001) regions. *L. rhamnosus* GG MTCC 1408 supplements attenuated the elevated PC levels in Ct (A5+Lb: 38.84%, p<0.01; A10+Lb: 32.24%, p<0.01) and Cb (A10+Lb: 34.38%, p<0.01). Furthermore, supplementation of *E. faecium* NCIM 5593 to ACR exposed mice could reduce PC levels in Cb (A10+EF: 23.97%, p<0.05) and Hc (A5+EF: 26.39%, p<0.05) regions (Figure 6).

Significant depletion in GSH levels was evident in ACR administered mice in Ct (A5: 37.51%, p<0.01; A10: 52.30%, p<0.01), Cb (A5: 28.26%, p<0.05; A10: 44.24%, p<0.01) and Hc (A5: 30.49%, p<0.01; A10: 41.96%, p<0.01) regions (Figure 5b and 7). While in Cb (A5+EF: 27.90%, p<0.05; A10+Lb: 18.30%, p<0.05) and Hc (A5+Lb: 21.27%, p<0.05; A5+EF: 24.82%, p<0.05; A10+Lb: 43.92%, p<0.01; A10+EF: 39.25%, p<0.01), *L. rhamnosus* GG MTCC 1408 and *E. faecium* NCIM 5593 exhibited significant increase in GSH levels. However, GSH levels were enhanced in Ct (A10+EF: 32.82%, p<0.01) region only by supplements of *E. faecium* NCIM 5593 (Figure 7).

Enzyme activity assays

L. rhamnosus GG MTCC 1408 and *E. faecium* NCIM 5593 supplements *per se* didn't alter CAT activity in Ct and Hc regions. Conversely, they enhanced CAT activity in Cb (Lb: 30.43%, p<0.01; EF: 25.27%, p<0.05). ACR exposed mice showed significant decrees in CAT activity (Ct' A5: 46.34%, p<0.01; A10: 73.65%, p<0.001; Cb' A5: 62.50%, p<0.001; A10: 75.01%, p<0.001; Hc' A5: 55.26%, p<0.001; A10: 69.44%, p<0.001) in all brain regions.

GG MTCC 1408 (A5+Lb: 53.84%, p<0.01) and *E. faecium* NCIM 5593 (A5+EF: 57.14%, p<0.01; A10+EF: 56.52%, p<0.01) to ACR exposed mice enhanced CAT activity significantly in Cb. Furthermore, *L. rhamnosus* GG MTCC 1408 supplements could effectively increase CAT activity among ACR exposed mice in Ct (A10+Lb: 23.34%, p<0.05). No effect was evident in Hc region (Figure 8a).

No significant change was evident with respect to SOD activity

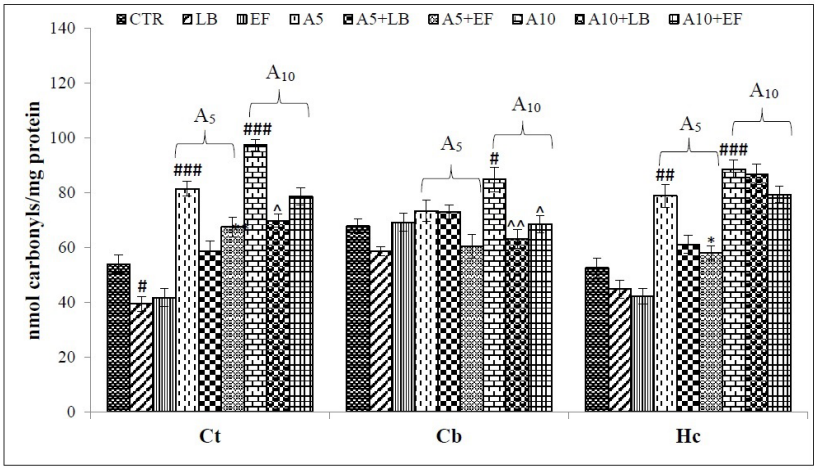

Figure 6: Effect of probiotic supplementation on PC levels.

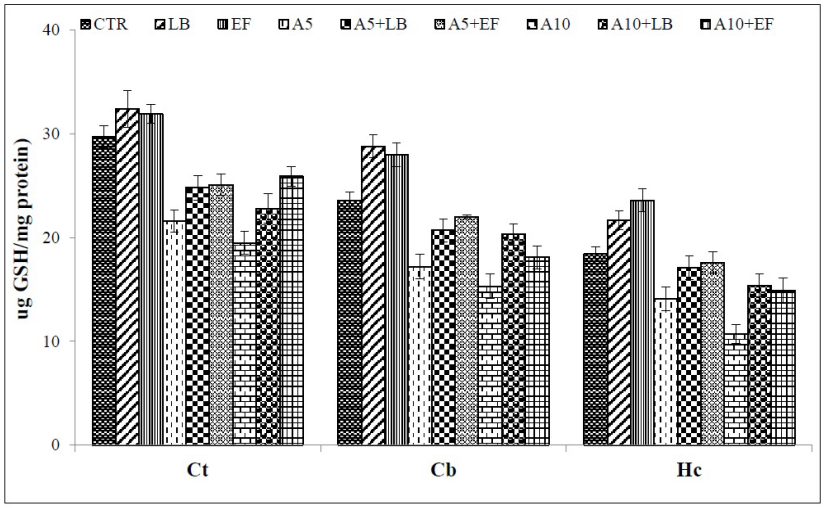

Figure 7: Effect of probiotic supplementation on GSH levels.

was singnificantly enhanced by *L. rhamnosus* GG MTCC 1408 and *E. faecium* NCIM 5593 supplemented mice in Cb and Hc regions (Figure 8b). However, *L. rhamnosus* GG MTCC 1408 and *E. faecium* NCIM 5593 supplements significantly enhanced SOD activity in Ct (Lb: 17.59%, p<0.05; EF: 13.32%, p<0.05). Significant change in SOD activity was observed in ACR administered mice in Ct (A10: 17.20%, p<0.05), Cb (A5: 26.57%, p<0.05; A10: 41.31%, p<0.01) and Hc (A5: 23.93%, p<0.05; A10: 47.64%, p<0.01) regions. ACR induced reduction in SOD activity was significantly enhanced *L. rhamnosus* GG MTCC 1408 (Cb' A10+Lb: 20.15%, p<0.05; Hc' A5+Lb: 14.28%, p<0.05; A10+Lb: 27.61%, p<0.05) and *E. faecium* NCIM 5593 (Ct'A10+EF: 22.12%, p<0.05; Cb' A5+EF: 12.66%, p<0.05; A10+EF: 15.74%, p<0.05; Hc' A5+EF: 12.47%, p<0.05; A10+EF: 25.10%, p<0.05) supplements in brain regions.

Acetylcholine esterase (AChE) activity

L. rhamnosus GG MTCC 1408 and *E. faecium* NCIM 5593 supplements *per se* didn't significantly alter AChE activity in all brain regions (Figure 9). Mice exposed to ACR at high dose was evident with marked increase in AChE activity in Ct (72.09%, p<0.001), Cb (49.31%, p<0.01) and Hc (67.27%, p<0.001) regions. Lower dose ACR enhanced AChE activity significantly only in Ct (28.32%, p<0.05) region of brain. *L. rhamnosus* GG MTCC 1408 and *E. faecium* NCIM 5593 supplementation could marginally reduce the elevated levels of AChE activity in all brain regions. However, the effect was significant in Ct (A5+EF: 25.58%, p<0.05) region of ACR exposed mice supplemented with *E. faecium* NCIM 5593.

Monoamine levels

In general, mice given *E. faecium* NCIM 5593 and *L. rhamnosus* GG MTCC 1408 *per se* showed enhanced cytosolic brain levels of monoamines in Ct (GABA' EF: 36.36%, p<0.01; DA' EF: 20.15%, p<0.05), Cb (GABA' EF: 13.34%, p<0.05; DA' EF: 10.06%, p<0.05; DA' Lb: 11.63%, p<0.05) and Hc (GABA' Lb: 25.34%, p<0.05; GABA' EF: 50.12%, p<0.01) regions (Figure 10a). However, *E. faecium* NCIM 5593 and *L. rhamnosus* GG MTCC 1408 supplements failed to enhance DA levels in Hc region (Figure 10b). ACR administrated caused marked decrease in GABA (Ct' A5: 69.23%, p<0.001; A10: 83.09%, p<0.001; Cb' A5: 75.12%, p<0.001; A10: 82.85%, p<0.001; Hc' A5: 50.12%, p<0.01; A10: 61.25%, p<0.001) and DA (Ct' A5: 41.35%, p<0.01; A10: 59.17%, p<0.01; Cb' A5: 42.84%, p<0.01; A10: 62.89%, p<0.001; Hc' A5: 38.78%, p<0.01; A10: 61.34%, p<0.001) levels in all brain regions in a dose dependent manner. Reduced levels of GABA were significantly enhanced by supplementation of *E. faecium* NCIM 5593 in Ct (A5+EF: 26.15%, p<0.05; A10+EF: 53.35%, p<0.01), Cb (A5+EF: 37.52%, p<0.01; A10+EF: 34.61%, p<0.01) and Hc (A5+EF: 28.57%, p<0.05;

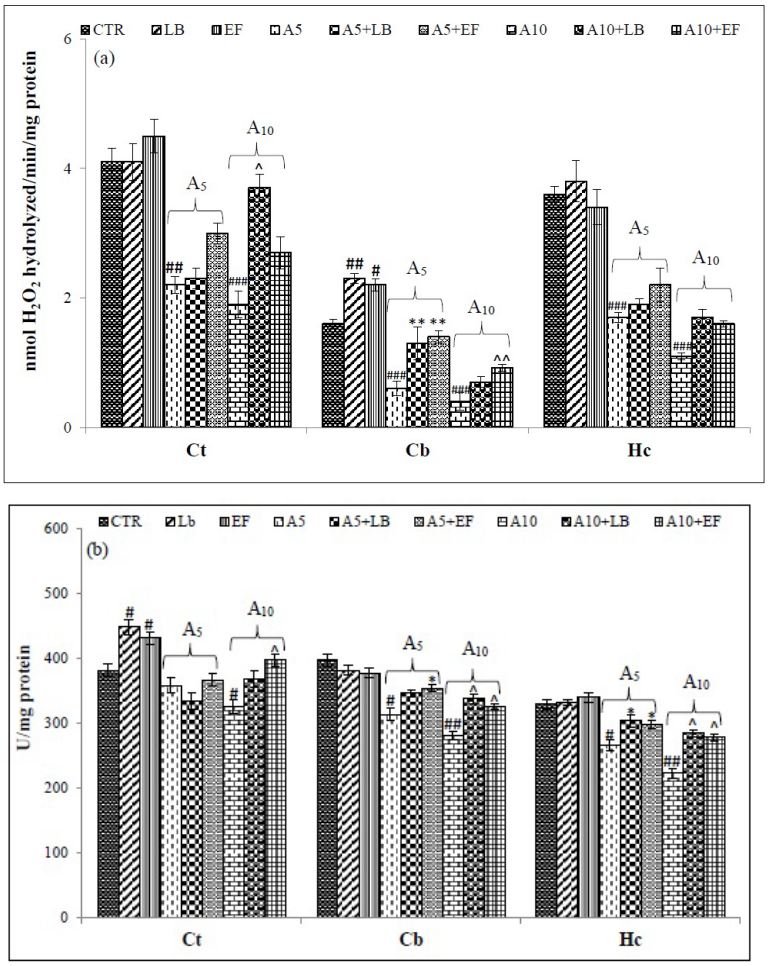

Figure 8: Effect of probiotic supplementation on enzyme activity (a) CAT (b) SOD.

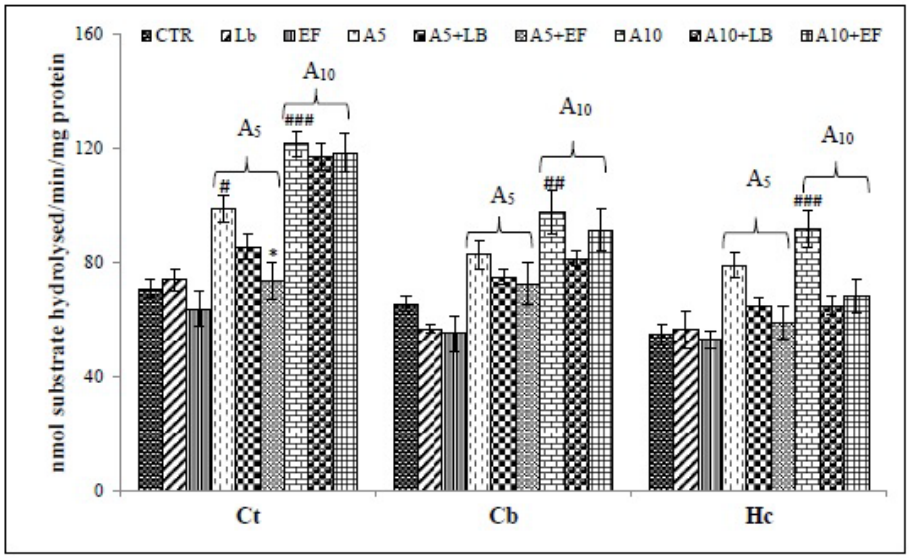

Figure 9: Effect of probiotic supplementation on AChE activity.

A10+EF: 40.38%, p<0.01) regions. In contrast, enhancement in GABA levels were observed for *L. rhamnosus* GG MTCC 1408 supplements in only Hc (A5+Lb: 28.12%, p<0.05) region. ACR induced reduction in DA levels were enhanced significantly in only Ct (A5+Lb: 23.32%%, p<0.05; A10+EF: 24.69%, p<0.05) and Cb (A5+EF: 19.67%, p<0.05) regions.

Discussion

Probiotics are increasingly used as a therapeutic agents in an ever-widening spectrum of diseases as well as in general health [27]. Probiotics plays a major role in the bidirectional communication between the gastrointestinal tract and the central nervous system (CNS) [28]. This ability of probiotics to communicate with the brain and to modulate behavior is now emerging as an exciting concept in health and diseases [29]. However, the routes of communication between the probiotics and brain are not fully elucidated. In recent years, numerous studies have demonstrated the importance of the gastrointestinal microbiota in the stress response [30,31] and in neurodevelopmental disorders [32-34]. In animal models, probiotic strains have proven successful in the treatment of mental health conditions. *B. infantis* 35624 demonstrated antidepressant properties [35], while *L. rhamnosus* JB-1 exhibited anti-anxiety and anti-depressant properties, compared to broth-fed control mice, through activation of the vagus nerve [6]. *B. fragilis* reduced autism-like behavioral deficits in communication, social behavior, and repetitive behaviour, in mice symptomatic of autism, compared to autistic, placebo treated controls [36].

E. faecium NCIM 5593 used in the study is a potent probiotic culture and known for its antioxidant and anti-inflammatory activity [9]. *E. faecium* NCIM 5593 has showed robust resistance to gastrointestinal stress conditions, demonstrating its ability to survive at various pH conditions, exhibited tolerance towards bile salts, pepsin and trypsin [9]. In addition, *E. faecium* NCIM 5593 demonstrated better viability following exposure to simulated gastric and intestinal conditions [23]. Furthermore, in our preliminary data obtained in whole-genome sequencing study, we found no evidence of virulent genes encoding gelatinase, hyaluronidase and cytolysin proteins which are associated with pathogenic *Enterococcus spp.* In addition, young mice provided with oral supplements of *E. faecium* NCIM 5593 exhibited diminished oxidative markers in the brain and enhanced activities of antioxidant enzymes with a concomitant increase in gamma-aminobutyric acid (GABA) and dopamine (DA) levels [9]. Thus, our strain showed its propensity to protect against tissue damage mediated through free radicals confer a neuroprotective advantage *in vivo* against oxidative damage-mediated neurodegenerative conditions. In line with previous study, the present investigation was sought to examine effect of *E. faecium* NCIM 5593 in mitigating neurotoxin induced oxidative dysfunctions and neurotoxicity.

GABA is the chief inhibitory neurotransmitter in the brain that regulates many physiological and psychological processes. Dysfunction in the GABA system is implicated in anxiety and depression [37,38]. Strains of *Lactobacillus, Bifidobacteria, Enteroccous and Streptocoocus* have shown their ability to produce GABA from monosodium glutamate *in vitro* [39-42]. It is been proposed that microbially produced GABA in gut may have profound effect on brain through gut-brain axis. Administration of GABA producing strain, *L. brevis* FPA3709 to rats had shown antidepressant effect similar to that of fluoxetine, a common antidepressant drug, but without the side-effects such as appetite and weight loss [43]. In the present study, a non-GABA producing strain, *L. rhamnosus* GG MTCC 1408 was also used to make better comparison between GABA producing and non-producing strains.

Acrylamide (ACR) is a well-known neurotoxin and one of the most important food contaminant occurring in heated food products. Accumulating evidence indicates that ACR induced toxicity is associated with oxidative stress and altered neurochemical balance. In the present study, ACR administration caused significant increase in anxiety-like behavior as evident by open field test and elevated plus maze. Furthermore, ACR administration caused significant oxidative damage as evident by increased ROS, MDA, NO, PC, and GSH levels in Ct, Cb and Hc regions of mice brain clearly stating that these tissues were subjected to significant oxidative stress *in vivo*. It also caused significant depletion in in the activities of antioxidant enzymes (SOD and CAT) and altered AChE activity in all brain regions. These findings are in line with Shinomol et al. [44] and Prasad and Muralidhara [45].

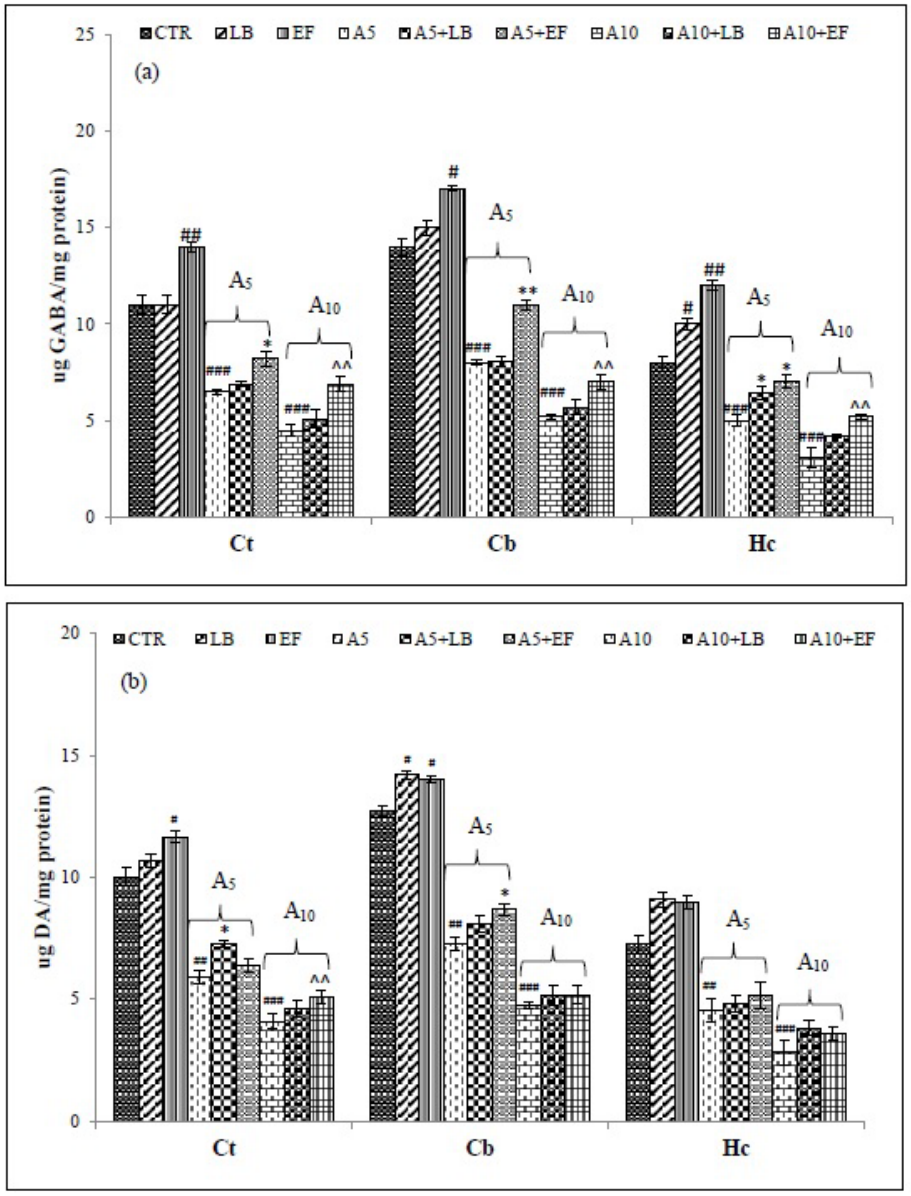

Figure 10: Effect of probiotic supplementation on monoamine levels. (a) GABA (b) DA.

Having established the *in vitro* antioxidant and *in vivo* neuromodulatory activities of *E. faecium* NCIM 5593 in our previous study, we employed these activities to ameliorate ACR induced oxidative stress.

The results obtained in the elevated plus maze test among mice given *E. faecium* NCIM 5593 and *L. rhamnosus* GG MTCC 1408 supplements are in line with previous reports on the potential of probiotics to reduce an anxiety-like response in animal models [6,9], which may be mediated through modulation of synaptic transmission as speculated previously [46]. All these findings suggest the psychobiotic potential of probiotics as speculated by Savignac et al. [47]. Nevertheless, comprehensive studies are further required to gain insights into the role of bacteria in modulating behavioural phenotypes.

E. faecium NCIM 5593 could significantly reduce ROS levels in Ct and Hc regions of normal mice brain as evidenced in our study [9]. Both *E. faecium* NCIM 5593 and *L. rhamnosus* GG MTCC 1408 reduced the ACR induced elevation of ROS levels, which is attributed to their free radical scavenging activity. LP is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity of many xenobiotics [48]. The prevention of lipid peroxidation is essential for all aerobic organisms and so the organism is well balanced with antioxidants that directly or indirectly protect cells against the adverse effects of xenobiotics and toxic radicals [49]. Interestingly, elevated levels of MDA were significantly reduced by *E. faecium*

NCIM 5593 and *L. rhamnosus* GG MTCC 1408 only in Ct region. Is worth noting that the greater reduction in MDA levels was observed for *E. faecium* NCIM 5593. Furthermore, *E. faecium* NCIM 5593 and *L. rhamnosus* GG MTCC 1408 supplementation to mice could reduce ACR induced elevation of MDA levels. There are no scientific evidence on the mechanism by which probiotics reduce

MDA levels in brain. However, administration of Probiotic yoghurt containing *L. acidophilus* La5 and *B. lactis* Bb12 to Type 2 diabetic patients could significantly reduce serum MDA concentration [50]. A decrease of MDA levels were observed in rats fed with *L. casei* Zhang [51].

Furthermore, the enhanced NO levels among ACR administered mice were also significantly diminished to the greater extent by the supplementation of *E. faecium* NCIM 5593. Although the activity of inducible NO synthase (iNOS) was not measured in this study, we speculate that the effect could be due to its enzyme activity inhibition by these organism. PC represent an irreversible form of protein modification and have been demonstrated to be relatively stable in contrast to LP products that are removed within minutes [52]. Moreover, PC are formed early during oxidative stress conditions and are not a result of one specific oxidant, thus they can be called a marker of overall protein oxidation [53]. Reduction in the levels of PC by *E. faecium* NCIM 5593 and *L rhamnosus* GG MTCC 1408 are in line with our previous study [9]. GSH (L-gamma-Glu-L-Cys-Gly) is a major cellular non-enzymatic antioxidant. Antioxidant probiotics has proven its ability to synthesize and handle GSH [54], which may be attributed to the increase in GSH activity in all brain regions.

AChE is a biological membrane component that contributes to its integrity and changes in permeability occurring during synaptic *transmission* and conduction [55]. AChE over activation leads to faster degradation of acetylcholine and consequent lowered stimulation of acetylcholine receptors, which causes a reduction of diverse cholinergic (learning, memory) and non-cholinergic (cell proliferation, neurite outgrowth) function [55,56]. Consequently, decrease in acetylcholine levels in the synaptic cleft contributes to a progressive cognitive impairment and neurodengerative disease [55,57]. In the present model, ACR induced significant elevation in the AChE activity in all brain regions and supplementation with *E. faecium* NCIM 5593 and *L rhamnosus* GG MTCC 1408 significantly restored the activity levels suggesting improvement in the acetylcholine mediated function. In line with these findings, studies have reported that certain *Lactobacillus* spp. have shown their ability to produce neurometabolites such as acetylcholine and GABA leading to enhancement in cholinergic neurotransmission [58]. Furthermore, probiotics influence amino acid metabolism and play an important role in neurotransmitter regulation, owing to their ability to produce neurometabolites [59], which is attributed to the presently observed enhanced levels of monoamines (GABA and DA) and probiotic supplements. Enhancement in GABA levels in ACR treated mice were observed only in the groups supplemented with *E. faecium* NCIM 5593, which could be attributed to its *in vivo* GABA production activity [23].

Conclusion

Increasing evidence now supports the role of probiotics in influencing various psychotropic behavioural aspects in animals [7]. Improvement in behavioral responses among probiotic treated mice further support to their neuroprotective efficacy. Although further studies are necessary to understand the underlying mechanisms of neuroprotection, we speculate that the antioxidant property of these probiotic strain be responsible for the protective effect. Enhancement in monoamines levels is highly attributed to the *in vivo* GABA production by *E. faecium* NCIM 5592. Thus, it could practically assumed that GABA producing probiotics administered chronically may provide viable approach to enhance neurochemical production *in vivo* and thus may serve as an innovative approach for clinical application.

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