Chapter 2

Evaluating the protective effect of probiotic *E. coli 16* on 1, 2-di-methyhydrazine (DMH) induced oxidative stress.

2. Introduction

Colorectal cancer is one of the most frequent types of malignant neoplasm in the world associated with high mortality rate and is the pathological consequence of persistent oxidative stress which is hazardous to health **(Schulmann et al., 2002)**. High levels of reactive oxygen species (ROS) is associated with cellular and mitochondrial damage and causes inflammation leading to tissue damage **(Roberts et al., 2010)**. Pathogens and commensal bacteria generate free radicals and may cause inflammatory bowel disease (IBD) **(Sekirov et al., 2010; Swanson et al., 2011)**. Antioxidant defence systems consisting of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) protect tissues from oxidative damage **(Choi et al., 2008)**. Under normal physiological condition, there is continuous generation of free radicals and other oxidants in all cells **(Moro et al., 2005)**. However, elimination rate of these free radicals in normal state is sufficient for maintaining oxidant homeostasis. Reactive oxygen and nitrogen species such as superoxide radical anion, hydroxyl radical, singlet oxygen, nitric oxide, and peroxy-nitrite can be generated by numerous forms of environmental stresses and neuronal insults. Effect of air pollutants is of prime interest within the scientific and regulatory community as it can be attributed to health effect like cardiovascular disease, respiratory disorder and oxidative stress related disorders **(Yang et al., 2009)**. During reduction of molecular oxygen to water in electron transport chain, reactive oxygen species (O_2^-, H_2O_2, OH•) are produced which can damage cellular systems. Cells prevent damage caused by these reactive oxygen species by defence mechanisms of antioxidants enzymes like Superoxide dismutase (SOD), catalase (CAT) which removes free radicals **(Harzallah et al., 2012)**. Brain makes up 2% of the body weight and it consumes approximately 20% of the body's energy and hence it is most vulnerable to destruction by oxidative damage. Oxidative stress plays a very important role in neurodegenerative diseases which could be correlated with alteration in neurotransmitter status **(Jellinger et al., 2010)**. Numerous antioxidants and herbal extracts have been extensively investigated as potential agents to ameliorate oxidative stress in the gastrointestinal tract and liver, i.e. butylated hydroxyl anisole (BHA), tert-butylhydroquinone (TBHQ), α-tocopherol,

vitamin C **(Shahidi et al., 2005)**. However, their effectiveness is limited by instability and ability to cause mutations leading to tumor formation.

Pyrroloquinoline quinone (PQQ) is highly a water-soluble, heat-stable molecule. In addition, it can undergo 20,000 redox catalytic cycles, whereas ascorbate and quercetin have the redox cycles of 4 and 800, respectively **(Rucker et al., 2009; Misra et al., 2012)**. PQQ has an antioxidant activity at <50 μM, and it has been shown to protect neuronal, liver and cardiac tissues against oxidative stress. Moreover, PQQ efficiently protects tissues against oxidative stress as compared to α-tocopherol and vitamin C **(Debray et al., 2008)**. PQQ plays an important role as an antioxidant in neuronal cells, enhances their regeneration and prevents death **(Nunome et al., 2008).** Nutritional studies on rodent models suggest that PQQ serves as growth factor, helps in development and improves reproductive parameters **(Rucker et al., 2009)**. Lack of PQQ in the diet causes decrease in learning ability, memory function and relative mitochondrial content **(Ohwada et al., 2008)**. PQQ can interact with brain neurotransmitter system and it protects neurons by modifying excito-toxicity which is mediated by powerful NMDA receptors in response to long term overstimulation of neurons leading to seizures and many neurodegenerative diseases **(Dong et al., 2009)**.In addition to acting as antioxidant, PQQ enhances mitochondrial biogenesis mediated by Janus kinase/signal transducer and activator of transcription (JAK/STAT) and mitogen activated protein kinase (MAPK) pathways leading to the expression of PGC-1α **(Chowanadisai et al., 2010; Tchaparian et al., 2010)**.

Oral administration of probiotics and prebiotics are known to decrease intestinal inflammation **(Ewaschuk et al., 2006)**. Some probiotics have been shown to have antioxidant properties which provide a protective effect in the pathogenesis of radiation-induced enteritis and colitis **(Spyropoulos et al., 2011)**. *Escherichia coli* Nissle 1917 has been shown to ameliorate intestinal epithelial cell damage in IBD chiefly by up-regulation of ZO-1 expression **(Ukena et al., 2007)**. *E. coli* CFR 16 is rats intestinal isolate having potential probiotic properties **(Kumar et al., 2009)**. In addition, recombinant *E. coli* CFR 16 strain is able to secrete inulosucrase enzyme possibly mediated by colicin E1/la1b transport system (**Kumar et al., 2013)**. Efficacy of probiotics as therapeutic agents against oxidative damage and inflammation is determined by their colonization and survivability **(Culligan et al., 2009)**. *Vitreoscilla*

hemoglobin (Vhb) improves cell survival, cell growth and antioxidant status **(Geckil et al., 2003)**. Colon cancer is the second to third most frequent type of cancer across the globe **(Schulmann et al., 2002)**. Rodent model of 1, 2-dimethylhydrazine (DMH)-induced colon cancer has been extensively used to study the efficacy of various antioxidant compounds on colon oxidative carcinogenesis and systemic oxidative stress **(No et al., 2007)**. DMH is also an environmental pollutant involved in causing oxidative stress in red blood cells by undergoing oxidative metabolism; as a consequence electrophilic diazonium ions are produced **(Fiala et al., 1978)**. DMH administered subcutaneously is slowly released in the circulation and it is metabolized to azoxy-methane and methylazoxy-methanol (MAM) in liver. MAM decomposition give rise to methyl diazonium ions generating carbonium ion as MAM is chemically unstable at body temperature. DMH is also known to cause neurotropic effects by altering brain neurotransmitter status and it could probably lead to convulsions and seizures **(Arutjunyan et al., 2001)**. Seizures are reported to be associated with altered redox status of the body and it can also alter neurotransmission attributing to neuronal cell death **(Andersson et al., 2004)**. Current study has been performed to evaluate the protective efficacy of probiotic *E. coli* CFR 16::*Vitreoscilla* hemoglobin gene (*vgb*)-green fluorescent protein (*gfp*) gene producing PQQ against DMH-induced oxidative damage.

2.1 Material and Methods

2.1.1 Animals

Adult Charles foster male albino rats (weight 200–250 g) were maintained in controlled temperature (25±1 °C), relative humidity (45.5 %), photoperiod cycle (12:12 h light/dark), food and free access to water as per recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines of Animal Ethical Committee of the Institute (M. S. University of Baroda, India, registration number 938/A/06/CPCSEA).

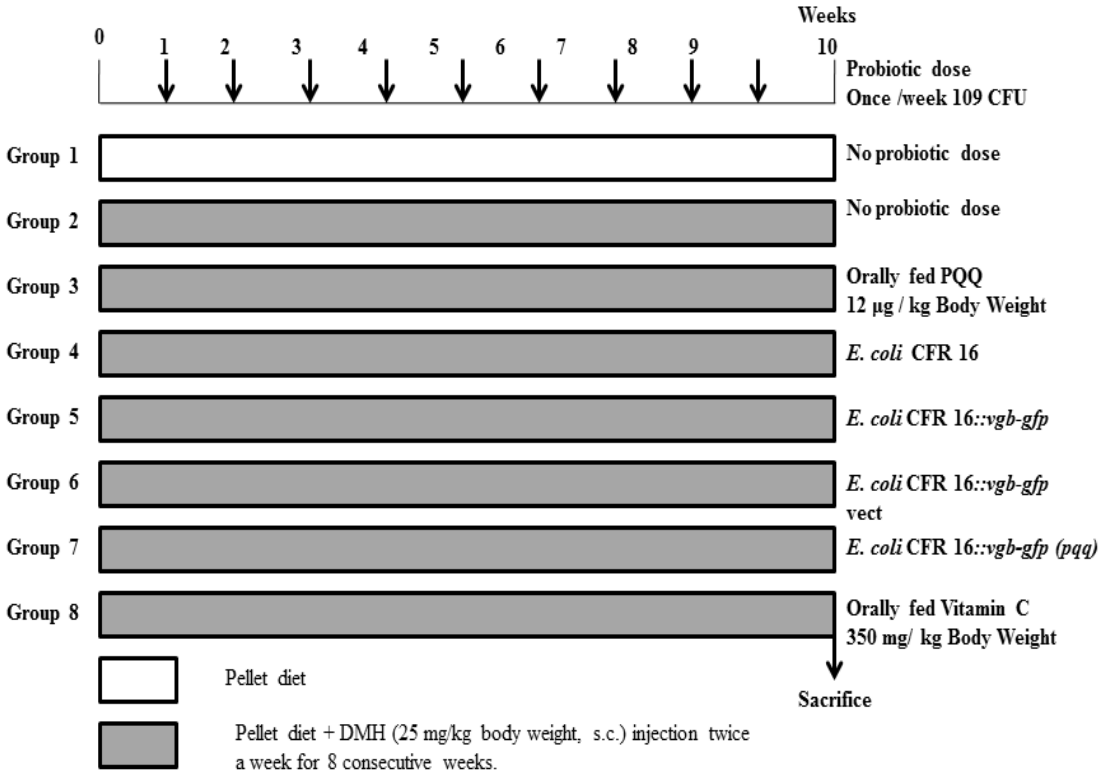

Fig. 2. 1: Schematic representation of the work plan of the experiment.

2.1.2 Experimental Design

For the present study, animals were divided into eight different groups (six animals per group) as follows: all rat groups received normal pellet diet. Apart from this, group 1 received 1 mM EDTA (pH 6.5) as vehicle and served as control group, group 2 received DMH (25 mg/kg body weight, s.c.) twice a week for 8 weeks, group 3 received DMH with orally fed PQQ (12 µg/kg body weight (BW)) once per week,

group 4 received DMH and E. coli CFR 16 probiotic once per week, group 5 received DMH and *E. coli* CFR 16:: *vgb-gfp* genomic integrant once per week, group 6 received DMH and *E. coli* CFR 16:: *vgb-gfp* vector control once per week, group 7 received DMH and E. coli CFR 16:: *vgb-gfp (pqq)* gene cluster once per week, and group 8 received DMH along with standard antioxidant vitamin C (350 mg/kg BW) once per week. Experimental protocol is shown in **Fig. 2.1.**

2.1.3 Plasmids and Constructs

Plasmid / strains	Characteristics	Reference
Plasmids		
pBBR1-MCS2	pBBR1-MCS2 Broad-host-range plasmid, *lacI*$^-$, multiple cloning site in *lacZ*; *Kan*^{r}	(Kovach et al., 1995)
pOK51	13.3-kb *Bam*HI fragment from pOK40 cloned into pBluescript II SK$^+$	(Choi et al., 2008)
pBBR1-MCS2*pqq*	pBBR1-MCS2 containing *Pseudomonas fluorescens* B16 pqqABCDE gene cluster at *BamHI* site	This study
Bacteria		
***Enterobacter asburiae* PSI3**	PQQ producing strain	(Gyaneshwar et al., 1998)
***E. coli* CFR 16**	Probiotic	(Kumar et al., 2009)
***E. coli* CFR 16::vgb-gfp**	*E. coli* CFR 16 containing *vgb* and *gfp* genes in the genome.	This study
***E. coli* CFR 16::vgb-gfp pBBR1-MCS2**	*E. coli* CFR 16::*vgb-gfp* with pBBR1-MCS2 plasmid	This study
***E. coli* CFR 16::vgb-gfp (pqq)**	*E. coli* CFR 16::*vgb-gfp* with pBBR1-MCS2 *pqq* plasmid	This study

Table 2. 1: Bacterial strains and plasmid.

All plasmids and bacterial cultures used in the present study are listed in **Table 2.1**. pOK51 plasmid containing a 13.3-kb *Pseudomonas fluorescens* B16 *pqq* gene cluster in Bam HI was received as a generous gift from Dr. Choi, Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul, South Korea. This gene cluster was sub-cloned in *Bam* HI site of pBBR1-MCS2 vector in our laboratory and was used for the experiments. pGRG36 vector was used for the genomic integration of *vgb-gfp* by Tn7-mediated integration system. Genomic integrants were confirmed by PCR and GFP fluorescence.

2.1. 4 Bacterial Strains and Culture Conditions

Probiotic *E. coli* CFR 16 strains containing different gene clusters were grown in Luria Broth (LB) overnight at 37 °C followed by re-inoculation in fresh medium to achieve final colony forming unit (CFU) of 10^9 cells/ml culture. One milliliter of this culture was taken out from the tube, centrifuged and washed twice with saline before tube feeding to different rat groups receiving probiotic dose.

2.1.5 PQQ Extraction and Quantification

PQQ was extracted from *Enterobacter asburiae* PSI3 by using a modified protocol of Suzuki **(Suzuki et al., 1990)**. In brief, *E. asburiae* PSI3 cells were harvested from overnight grown culture in M9 minimal medium, and the culture supernatant was harvested in fresh tube. Culture supernatant was treated by addition of 50 % acetonitrile at 65 °C for 2 h followed by centrifugation at 15,000g for 10 min. Clear supernatant was collected and dried with a concentrator under vacuum. The residues obtained were dissolved in 50 % n-butanol at 1 mg/ml, and the PQQ was extracted by overnight incubation at 50 °C. Quantification of PQQ was carried out fluorimetrically at excitation of 340-360 nm and emission of 418 nm. Extraction and quantification of PQQ from rat fecal sample was also carried out using the same protocol. In brief, 1 g of fresh fecal sample was collected in a 50-ml tube followed by addition of chilled Milli-Q water and vigorous vortexing and was centrifuged at 1,000 rpm for 5 min, and the supernatant was collected. So, supernatant obtained was further processed similar to the culture supernatant as described above.

2.1.6 Preparation of Tissue Homogenates

At the end of 10th week, blood was collected in EDTA vials and they were sacrificed by decapitation for estimation of other tissue parameters. Whole brain was removed quickly, washed with PBS and it was immediately used for neurotransmitter estimation. LPO and antioxidant enzyme activities were performed from pack cell volume (PCV). Liver and colonic tissues were removed immediately after sacrificing rats followed by washing with ice-cold saline before homogenizing it in appropriate buffer.

2.1.7 Serum glutamate pyruvate transaminase (SGPT) and serum glutamic oxaloacetate transaminase (SGOT) estimation

SGOT and SGPT levels in serum were measured by kit from Coral, Tulip group of companies, Goa, India as per manufacturer protocol.

2.1.8 Estimation of Norepinephrine (NE) and Dopamine (DP)

Norepinephrine and dopamine were estimated by fluorimetric method **(Jacobowitz et al., 1978)**. In brief, whole brain was homogenized in 5 ml of butanol and 0.75 ml of 0.01N HCl followed by centrifugation at 1500g for 10 minutes at 4˚C. Butanol layer was then transferred to a tube containing 3 ml of 0.1M phosphate buffer (pH-6.5). NE and DA were extracted in to phosphate layer and then centrifuged at 3000g for 5 minutes to separate the organic layer from aqueous phase. 1 ml of phosphate layer was transferred to a tube containing 0.25 ml of versene and vortexed briefly. After 2 minutes interval, 0.2 ml of iodine solution was added followed by addition of 0.25 ml of freshly made alkaline sodium sulphite. Add 0.3 ml of 5N acetic acid and shake well. Keep the test tubes in boiling water bath for 5 minutes and cool it on ice. NE was measured immediately at 485 nm by giving prior excitation at 385nm. After 20 minutes DA was estimated from the same samples by exciting at 320nm and measuring emission at 370nm.

2.1.9 Estimation of 5-Hydroxy tryptophan (5-HT)

Serotonin estimation was carried out by the method of Curzon **(Curzon et al., 1970)**. Brain was homogenized in 3 ml of acidified butanol followed by centrifugation at 1500 g for 10 minutes to settle down the tissue debris. 2.5 ml of the supernatant was mixed with 5 ml of n-heptane and 0.6 ml of 0.1 % cysteine in which serotonin was extracted and the two layers were separated by briefly centrifuging at 3000g for 5 minutes. 0.2 ml of the aqueous layer was transferred to a tube containing 0.8 ml of freshly prepared o-phthalaldehyde solution and 0.02 ml of 1 % cysteine. The tubes were placed in 77 ˚C water bath for 15 minutes and 5-HT florescence was measured at 370/480 nm with the help of spectro-flourimeter.

2.1.10 Estimation of Epinephrine

Epinephrine was estimated according to the method of **Ghosh et al., 1951**. 20 % tissue homogenates were made in 10 % trichloroacetic acid followed by centrifugation at 10,000 rpm for 10 minutes at 4 ˚C. 0.2 ml of supernatant was added to a tube containing 0.25 ml of 10 % sodium carbonate and incubated for 30 minutes at room temperature followed by addition of 125 µl of Folin's reagent and 375 µl of 5 % NaOH. Absorbance of Epinephrine was recorded at 486 nm within 1.5 minutes.

2.1.11 Superoxide Dismutase

Measurement of SOD activity was carried out using the method which is based on the capacity of pyrogallol to autoxidize, a process dependent on oxygen, which is a substrate for SOD **(Marklund et al., 1974)**. The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity can then be assayed spectrophotometrically at 420 nm. SOD activity of samples was calculated by using standard curve and was given as units per milligram of protein.

2.1.12 Catalase

Catalase was assayed by measuring the disappearance of hydrogen peroxide (H_2O_2) with a spectrophotometer at 240 nm **(Beers et al., 1952)**. One unit of the enzyme is defined as 1 *µmol* of hydrogen peroxide consumed per min, and the specific activity is reported as units per milligram of protein.

2.1.13 Glutathione Peroxidase Activity Assay

GPx was carried out using the method which is based on the ability of GPx to act on hydrogen peroxide in the presence of GSH, thereby depleting it; the remaining GSH is measured by the DTNB colour reaction reading at 412 nm. GPx activity was expressed as micromoles of GSH utilized per minute per milligram **(Beutler et al., 1963)**.

2.1.14 Histopathological Changes

After sacrificing, the colon tissues were fixed in 10 % buffered formalin, followed by hematoxylin and eosin staining. Histopathological examinations of all the tissues were performed by a pathologist unaware of the experimental codes. Crypt architecture, degree of inflammatory cell infiltration, muscle thickening, goblet cell depletion and crypt abscess were taken into consideration to examine the extent of oxidative damage.

2.1.15 Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using Graph Pad Prism 3.00 for Windows, Graph Pad Software. The statistical significance of the data was determined using one-way analysis of variance (ANOVA). The results were considered statistically significant at $P<0.05$.

2.2 Results

2.2.1 Characterization and Quantification of PQQ-Producing Probiotic *E. coli* CFR 16

P. fluorescens B16 *pqq* gene cluster of 13.3 kb was sub-cloned in pBBR1MCS2 vector. Upon confirmation of the recombinant plasmid by restriction digestion and PCR, the *E. coli* CFR 16:: *vgb-gfp* (*pqq*) transformant was generated. Functionality of PQQ-dependent glucose dehydrogenase activity of *E. coli* CFR 16:: *vgb-gfp* (*pqq*) was determined on Tris-buffered media with methyl red pH indicator **(Fig. 2.2)**. *E. coli* CFR 16:: *vgb-gfp* (*pqq*) showed red colour formation as an indicator of gluconic acid production which is absent in the vector control of *E. coli* CFR 16:: *vgb-gfp*. *E. coli* CFR 16:: *vgb-gfp* (*pqq*) produced 1.6 µg PQQ/ml of culture medium.

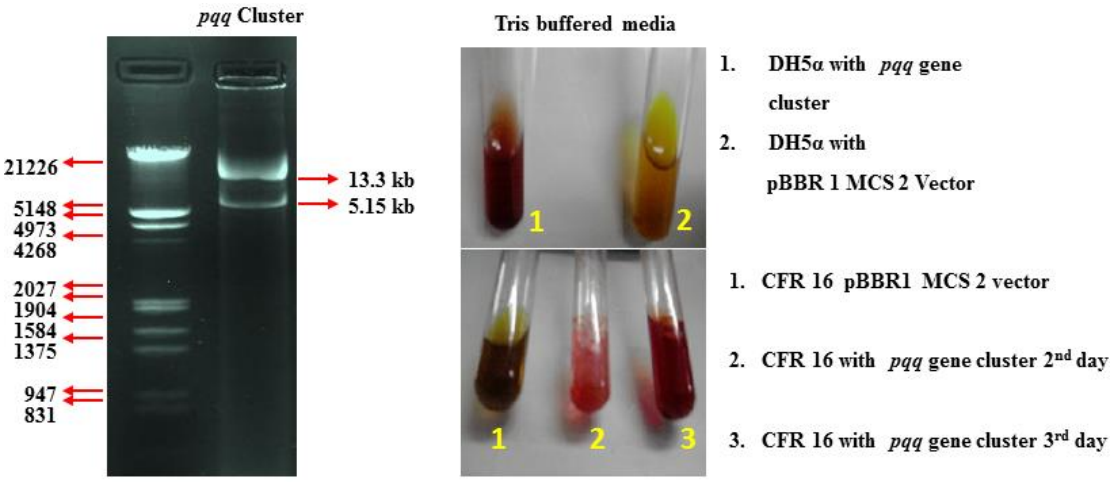

Fig. 2.2: Confirmation and functionality of *pqq* gene cluster.

2.2.2 Fecal PQQ Quantification

Fecal PQQ quantification of rat group administered with *E. coli* CFR 16:: *vgb-gfp* (*pqq*) was assessed to check the functionality of *pqq* gene cluster in vivo. Fecal PQQ concentration in rat group fed with *E. coli* CFR 16:: *vgb-gfp* (*pqq*) (group 7 rats) was 1.395 nmol/g which is almost twofold higher than that in control rat group **(Table. 2.2)**.

Control	DMH	Orally fed PQQ	*E. coli* CFR 16	*E. coli* CFR 16*::vgb-gfp*	*E. coli* CFR 16*::vgb-gfp* Vector	*E. coli* CFR 16*::vgb-gfp (pqq)*	Vitamin C
0.774 ± 0.021	0.669 ± 0.038	2.279 ± 0.131	0.725 ± 0.019	0.749 ± 0.026	0.770 ± 0.041	1.395** ± 0.121	0.719 ± 0.022

Table 2. 2: Fecal PQQ concentration of all rat groups at the end of the experiment.

2.2.3 Effect of Probiotic *E. coli* CFR 16 on Body Weight and Colon Length

Subcutaneous injection of DMH was tolerated by all rat groups as evident from a lack of any clinical signs of toxicity. Body weight profile of all rat groups did not show any significant changes in body weight except in group 2 rats. In addition, group 2 rats administered with DMH without any probiotic supplementation showed a significant reduction of colon length in comparison to all the other groups, suggesting that orally fed PQQ, wild-type *E. coli* CFR 16, *E. coli* CFR 16:: *vgb-gfp*, *E. coli* CFR 16:: *vgb-gfp* (*pqq*) as well as vitamin C were able to restore colon length significantly **(Fig. 2.4)**. Colon length of group 7 rats receiving *E. coli* CFR 16:: *vgb-gfp* (*pqq*) was found to be significantly higher in comparison to that of rat groups 4, 5 and 6 receiving wild-type *E. coli* CFR 16, *E. coli* CFR 16:: *vgb-gfp* and *E. coli* CFR 16:: *vgb-gfp* vector control, respectively. However, there was no significant difference observed among rat groups 3, 7 and 8.

2.3.4 Serum SGOT and SGPT

DMH is known to cause hepatic toxicity therefore we first monitored SGPT and SGOT levels as biomarkers of liver toxicity. Both serum SGPT and SGOT levels exhibited marked increase in Group 2 animals in comparison to Group 1 animals **(Table 2.3)**.

Groups	SGPT (IU/l)	SGOT (IU/l)
Control	26.43 ± 3.42	33. 12 ± 4.3
DMH	123. 65 ± 10.09***	126 .11 ± 11.22***
Orally fed PQQ	56.78 ± 6.77##	63 ± 5.22##
E. coli CFR 16	118.01 ± 13.22***	134. 33 ± 12.23***
E. coli CFR 16:: vgb-gfp	126.21 ± 9.90***	117 ± 10.23***
E. coli CFR 16:: vgb-gfp Vector	117. 20 ± 11.33***	124. 9 ± 8.99***
E. coli CFR 16:: vgb-gfp (pqq)	43. 65 ± 7.53##	42. 79 ± 6.87##
Vitamin C	90.21± 7.65**	95. 33 ± 8.56**

Table 2. 3 Serum SGOT and SGPT levels. Values are expressed as Mean ± SEM (n=6). ***P≤ 0.001, ** P≤ 0.01 in comparison to control rat group. ## P≤ 0.01 in comparison to Vitamin C group.

2.2.5 Effect of Probiotic *E. coli* CFR 16 on systemic antioxidant status

Systemic LPO is a marker for oxidative stress and displayed significant increase in group 2 animals. Elevated levels were restored near to normal levels in Group 3, 7 and 8 receiving Oral dose of PQQ, *E. coli* CFR 16::*vgb-gfp (pqq)* and Vitamin C, respectively. However, Group 4 did not show any change in LPO levels. Group 5 and 6 animals exhibited some degree of protection but it was less than Group 3, 7 and 8 animals **(Fig. 2.3).** Blood SOD activity was also found to be reduced in Group 2 animals as comparison to Group 1 animals. As observed in case of LPO levels, SOD activity was also restored to normal levels after oral PQQ and *E. coli* CFR 16::*vgb-gfp (pqq*) administration in Group 3 and 8 animals respectively. In this case, SOD activity was found to be significantly lower in Group 8 animals in comparison to Group 3 and 7 animals suggesting that probiotic producing PQQ show better protection against oxidative stress than Vitamin C.

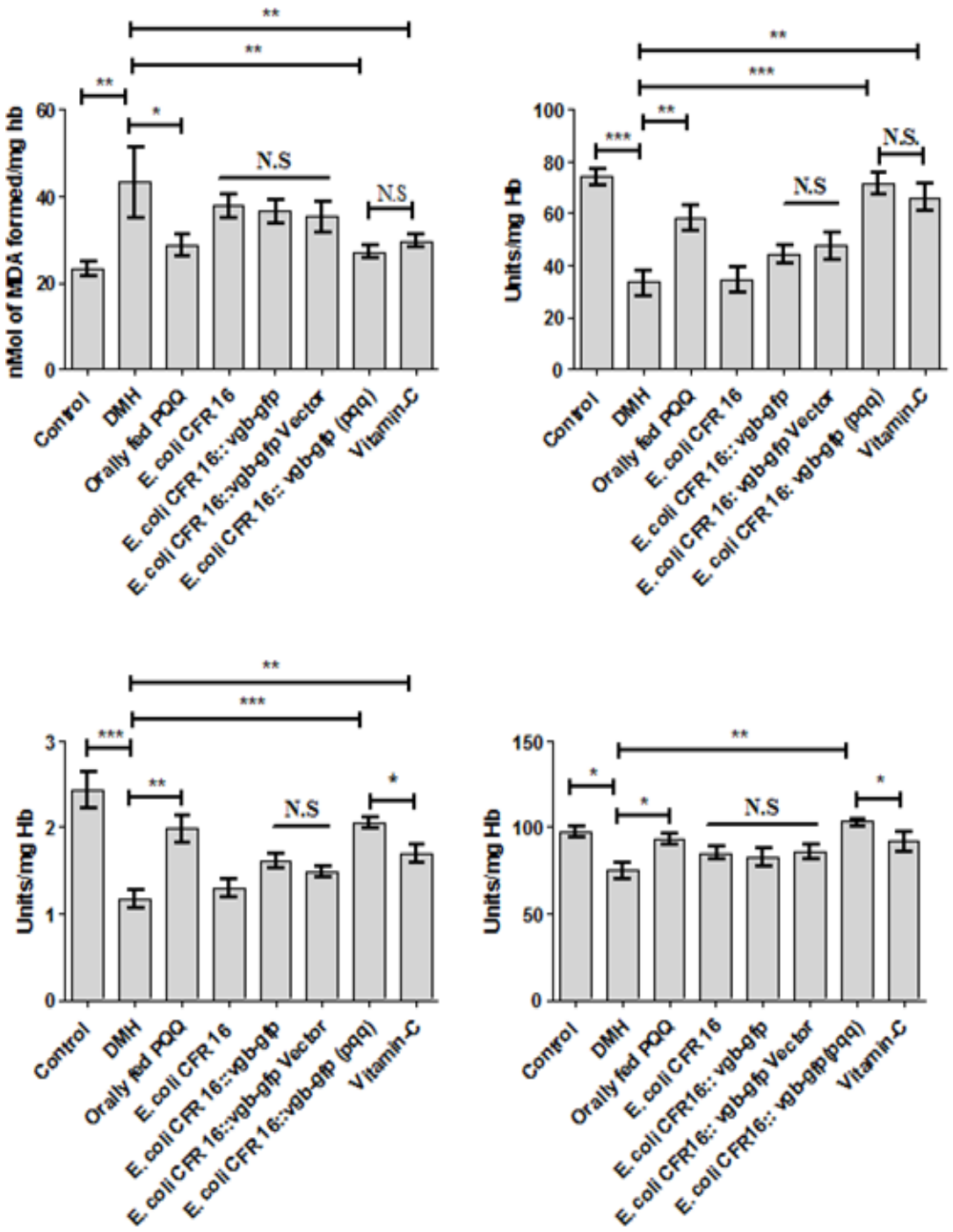

Fig. 2.3 Blood antioxidant status. (A) LPO; (B) CAT; (C) SOD and (D) G6PDH. Values are expressed as mean ± SEM (n=6 each group). *P≤ 0.05, **P ≤ 0.01 and ** P ≤ 0.001

2.2.6 Effect of Probiotic *E. coli* CFR 16 on Liver and Colon Lipid Peroxidation

To further investigate the oxidative stress induced by DMH at tissue level, liver and intestinal lipid peroxidation (LPO) levels were monitored as a marker of oxidative stress. Group 2 rats administered with DMH without any probiotic supplementation showed a higher level of lipid peroxidation in both tissues, indicating

severe oxidative stress **(Fig. 2.4)**. Supplementation with orally fed PQQ, wild-type *E. coli* CFR 16, *E. coli* CFR 16:: *vgb-gfp* and *E. coli* CFR 16:: *vgb-gfp* vector, *E. coli* CFR 16:: *vgb-gfp (pqq)* and vitamin C restored LPO levels significantly.

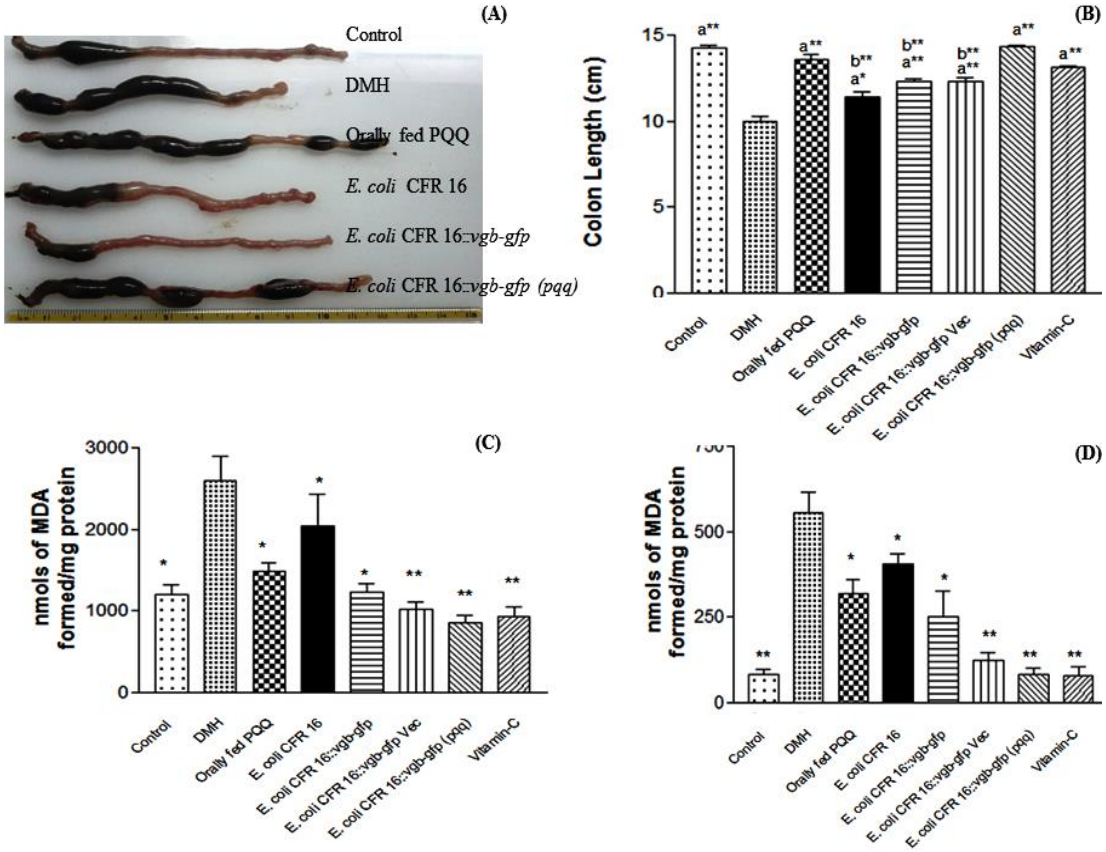

Fig. 2.4: Effect of probiotic on Length of colon (A and B) and Lipid peroxidation (Liver (C) and Colon (D)) in different groups treated with DMH after 10 weeks. Values are expressed as mean ± SEM (n=4 each group).a*P ≤ 0.01 and a P ≤ 0.001 compared with DMH group. b* P ≤ 0.01, b**P ≤ 0.001 compared with 16::vgb-gfp (pqq) group in (A) and (B). (C) and (D) values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.01 and ** P ≤ 0.001 compared with DMH group.**

Group 7 receiving *E. coli* CFR 16:: *vgb-gfp* (*pqq*) and group 8 receiving vitamin C showed improved protection in comparison to orally fed PQQ, *E. coli* CFR 16, *E. coli* CFR:: *vgb-gfp E. coli* and CFR:: *vgb-gfp* vector control rats. Moreover, groups 7 and 8 rats did not showed any significant difference in lipid peroxidation of liver and colon tissue.

2.3.7 Effect of Probiotic *E. coli* CFR 16 on Liver and Colon Antioxidant Status

Liver and colon antioxidant enzyme activities (SOD, CAT and GPx) were monitored to assess the antioxidant status at tissue level. SOD activity showed a

significant decrease in the liver tissue of group 2 rats administered with DMH without probiotic supplementation in comparison to control group. All other rat groups (orally fed PQQ, *E. coli* CFR 16:: *vgb-gfp* and *E. coli* CFR 16::*vgb-gfp* vector, *E. coli* CFR 16:: *vgb-gfp* (*pqq*) and vitamin C) showed a significant restoration of SOD activity except in group 4 receiving wild-type probiotic *E. coli* CFR 16. In addition, group 7 rats receiving *E. coli* CFR 16:: *vgb-gfp* (*pqq*) showed significantly higher restoration of SOD activity in comparison to groups 4, 5, 6 and 8 rats. Proximal and distal colonic tissue also showed a significant decrease in SOD activity of group 2 which were restored in all other groups. Here also, group 7 rats receiving *E. coli* CFR 16:: *vgb-gfp* (*pqq*) showed better protection in comparison with proximal colonic tissue of groups 4, 5, 6 and 8 rats **(Table 2.4)**.

Catalase activity showed a significant reduction in the liver tissue of group 2 receiving DMH treatment without probiotic supplementation, indicating very high oxidative stress. On treatment with probiotics, all groups orally fed with PQQ and vitamin C showed a significant increase in CAT activity. Moreover, group 7 rats receiving *E. coli* CFR 16:: *vgb-gfp* (*pqq*) showed better restoration than groups 4, 5 and 6 rats. There was no significant difference observed between groups 7 and 8. Similar pattern of CAT activity was observed in proximal as well as distal colonic tissue. Here also, group 7 showed better restoration than groups 4, 5 and 6, but no significant difference was observed in groups 7 and 8 **(Table 2.4)**.

GPx activity of group 2 rats also showed significant reduction in the liver and colon tissue on comparison with all other rat groups. Oral PQQ, probiotic and vitamin C supplementation significantly restored GRx activity in the liver, proximal colon and distal colon. Groups 3, 7 and 8 rats showed restoration up to normal levels, indicating better protection from oxidative stress in comparison to groups 4, 5 and 6 rats **(Table 2.4)**.

2.3.8 Colon Histological Analysis

Histological sections of colonic tissue were carried out by hematoxylin and eosin staining to check tissue architecture at cellular level.

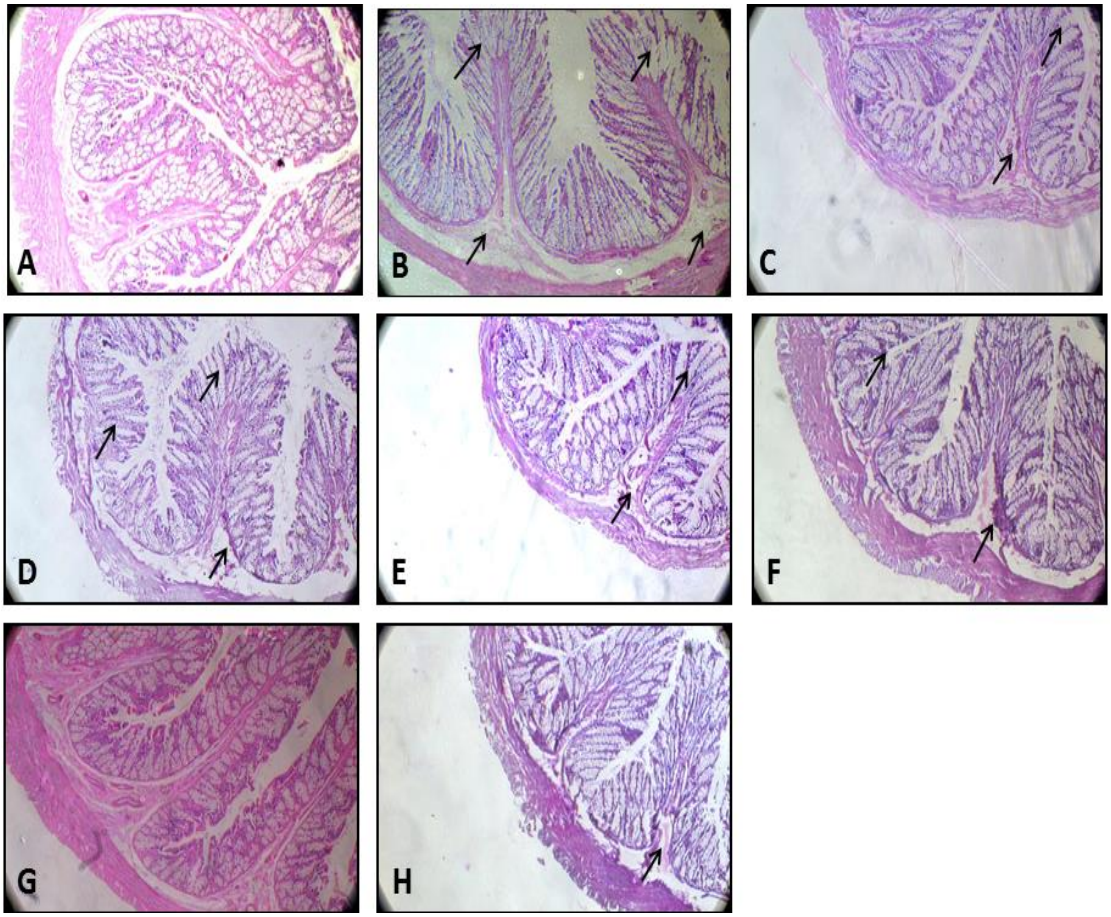

Fig. 2.5: Histology of colonic tissue in different groups. (A) Control (B) DMH (C) CFR 16 (D) CFR 16::*vgb-gfp* (E) Orally fed PQQ (F) Vitamin C (G) CFR16::*vgb-gfp* vector (H) CFR16::*vgb-gfp (pqq)*

	Control	DMH	Orally fed PQQ	CFR 16 Isolate	CFR 16::*vgb-gfp*	CFR 16::*vgb-gfp* Vector	CFR 16::*vgb-gfp* (*pqq*)	Vitamin-C
SOD (50 % alkaline pyragallol reduction/min/mg protein)								
Liver	7.29 ± 0.59	4.69 ± 0.33a*	6.50 ± 0.33ab	5.22 ± 0.62a*b*	6.14 ± 0.10a*b*	6.00 ± 0.18a*b*	7.12 ± 0.14	5.48 ± 0.13a*b
Proximal colon	6.13 ± 0.16	4.48 ± 0.26a*	5.43 ± 0.27b	5.10 ± 0.17a*b*	5.52 ± 0.16a*b*	5.50 ± 0.21a*b*	6.33 ± 0.22	5.58 ± 0.13ab
Distal colon	8.08 ± 0.24	5.36 ± 0.27a*	7.57 ± 0.35b	6.37 ± 0.33a*b*	7.17 ± 0.14a*b*	6.55 ± 0.24a*b*	7.95 ± 0.22	7.39 ± 0.24ab
CAT (µmol H_2O_2 utilized/min/mg protein)								
Liver	56.92 ± 3.07	28.21 ± 2.09a*	48.38 ± 2.58ab	35.1 ± 2.70a*b*	43.33 ± 2.28a*b*	37.92 ± 2.69a*b*	50.98 ± 2.95	47.60 ± 1.92ab
Proximal colon	64.09 ± 3.48	40.70 ± 3.14a*	57.76 ± 1.96b	50.06 ± 2.31a*b*	55.76 ± 2.41a*b*	52.43 ± 2.64a*b*	59.97 ± 1.69	55.28 ± 2.41ab
Distal colon	51.72 ± 3.03	25.40 ± 2.55a*	42.70 ± 1.98ab	35.19 ± 2.03a*b*	40 ± 2.44a*b*	35.25 ± 2.17a*b*	47.85 ± 2.18	42.25 ± 1.84ab
GPx (µmoles of GSH utilized/min/mg protein)								
Liver	6.59 ± 0.30	4.26 ± 0.22a*	5.95 ± 0.13b	4.95 ± 0.14a*b*	5.31 ± 0.14a*b*	4.96 ± 0.22a*b*	6.23 ± 0.18	5.84 ± 0.21b
Proximal colon	5.61 ± 0.23	3.27 ± 0.18a*	5.04 ± 0.14ab	4.52 ± 0.20a*b*	5.05 ± 01.6a*b*	4.75 ± 0.19a*b*	5.45 ± 0.21	5.09 ± 0.12ab
Distal colon	5.08 ± 0.13	2.33 ± 0.17a*	4.32 ± 0.20ab	3.53 ± 0.22a*b*	4.08 ± 0.13a*b*	4 ± 0.20a*b*	4.60 ± 0.13	4.24 ± 0.24ab

Table 2. 4: Effect of orally fed PQQ, different constructs of *E. coli* CFR 16 probiotics and Vitamin-C on SOD (Superoxide dismutase), CAT (Catalase) and GPx (Glutathione peroxidase) activity in control and experimental rats. Values are expressed as Mean ± SEM (n=6 each group). a $P \leq 0.05$, a* $P \leq 0.001$ compared with DMH group. b $P \leq 0.05$, b* $P \leq 0.001$ compared with 16*:: vgb-gfp (pqq)* group.

Colonic tissue of group 1 showed normal tissue architecture, whereas severe tissue damage (loss of crypt architecture, muscle disruption, infiltration of inflammatory cells) was observed in group 2 rats which served as DMH control group. Upon treatment with orally fed PQQ, group 3 showed much better protection, but prominent damage was seen in the peripheral region and muscularis. Group 4 receiving native probiotic *E. coli* CFR 16 treatment showed protection against oxidative stress albeit at a lesser extent which was further improved in group 5 that received probiotic *E. coli* CFR 16:: *vgb-gfp* supplementation. In comparison, group 7 showed better protection against oxidative stress, whereas group 8 receiving standard antioxidant, vitamin C had tissue damage which was prominent in muscularis region, indicating that PQQ-producing probiotic is more effective than orally fed PQQ and vitamin C **(Fig. 2.5)**.

2.3.9 Brain neurotransmitter status

Brain serotonin levels were found to be reduced in Group 2 whereas Group 3 and Group 8 rats exhibited near normal levels of serotonin. This indicates orally fed PQQ and PQQ producing *E. coli* CFR 16 can modulate brain neurotransmitter levels. In contrast, all the other groups did not show any change including Vitamin-C fed rats **(Fig. 2.7)**. This observation could be attributed to efficacy of PQQ to modify the metabolizing enzymes or reuptake transport proteins. However, this fact still remains to be elucidated. As balance between epinephrine and nor-epinephrine is crucial for maintaining mood homeostasis and stress regulation, we measured these levels in all the rat groups. Epinephrine content of Group 2 was found to be elevated and non-epinephrine levels were reduced in comparison to Group 1 rats. On supplementation with oral PQQ dose and PQQ producing *E. coli* CFR 16 these levels were restored to normal levels. However, Vitamin-C administration restored epinephrine levels but did not show any change in nor-epinephrine levels. As observed in case of serotonin here also all the other groups did not exhibited any change **(Fig. 2.6)**. Decrease obtained in DMH treated group could be related to the fact that neurotransmitters are auto-oxidisable. Monoamine- Serotonin, norepinephrine can react with O2 to generate superoxide radicals.

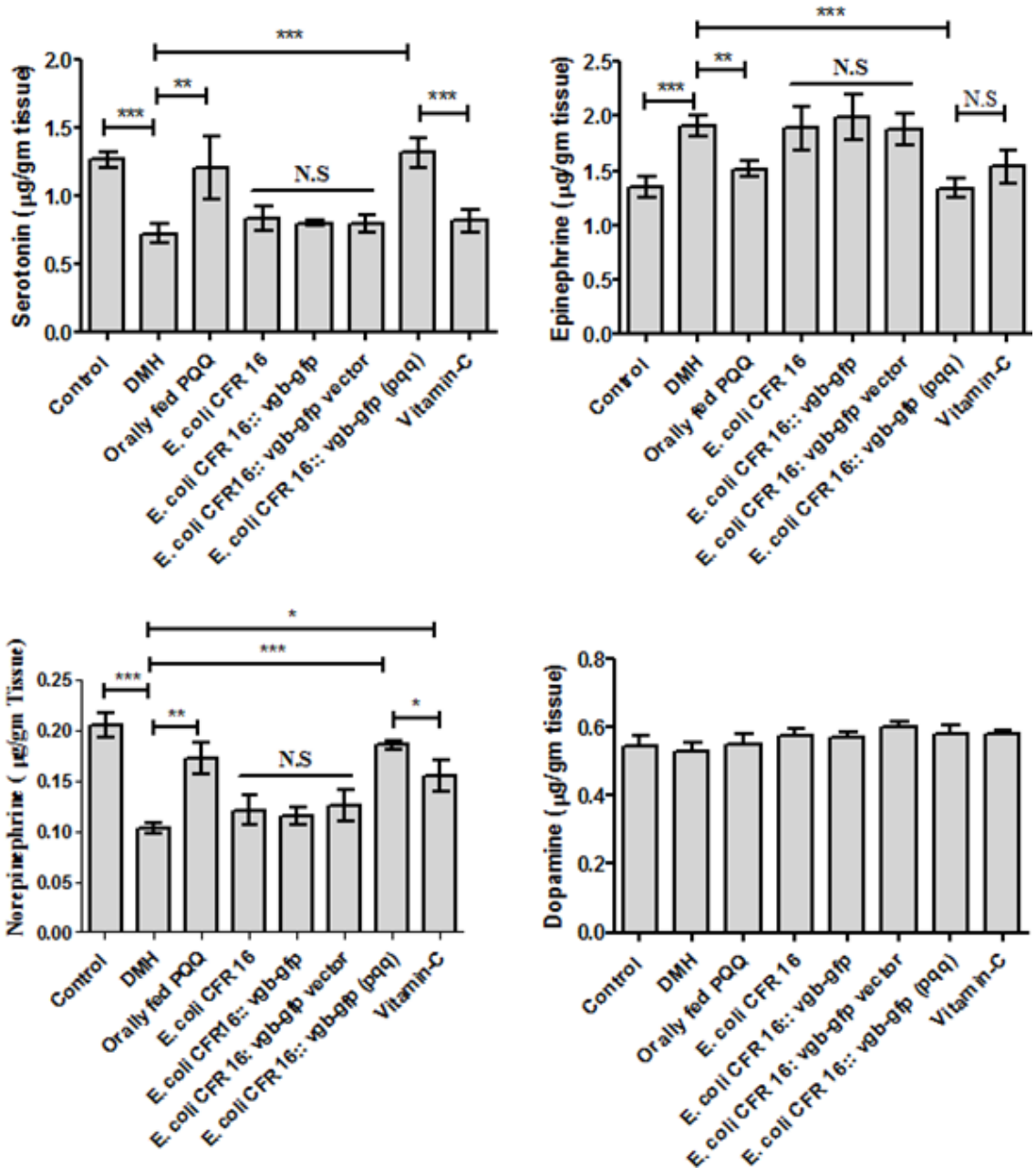

Fig. 2.6 Brain neurotransmitter status. (A) Serotonin; (B) Epinephrine; (C) Nor-epinephrine and (D) Dopamine. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, **P ≤ 0.01 and ** P ≤ 0.001.

2.3 Discussion

PQQ is present in a wide variety of foods and has gained considerable significance because of its remarkable antioxidant properties, heat stability and solubility in water **(Rucker et al., 2009; Misra et al., 2012)**. PQQ biosynthesis genes have been identified in various bacteria, including *Acinetobacter calcoaceticus, Methylobacterium extorquens* AM1, *Klebsiella pneumoniae, Gluconobacter oxydans, P. fluorescens* B16, *P. fluorescens* Pf0-1 and *P. fluorescens* CHA0 **(Choi et al., 2008; Misra et al., 2012)**. However, interestingly, intestinal microbiota does not appear to synthesize PQQ which is evident from the studies demonstrating developmental defects in rats fed with PQQ-deficient diet **(Rucker et al., 2009)**. Probiotic *E. coli* CFR 16 tagged with GFP estimated the rat intestinal colonization in the range of ~$10^{6–7}$, indicating that the genetic modifications have not diminished colonization ability. Increased level of PQQ in the fecal sample demonstrates the functionality of *pqq* gene cluster in in vivo conditions.

Human intestine is considered to be anaerobic in nature; therefore, aerobic bacterial respiration is essential for effective competition and colonization of *E. coli* in the micro-aerobic environment of intestine **(Backhed et al., 2005; Jones et al., 2011)**. Gene product of *vgb* gene, Vhb, has been demonstrated to improve growth and antioxidant property of bacteria **(Culligan et al. 2009)**. Intracellular expression of *vgb* gene in various heterologous hosts often results in the enhancement of cell density, oxidative metabolism, engineered product formation and bioremediation, especially under oxygen limiting conditions. In the present study, group 5 rats receiving *E. coli* CFR 16:: *vgb-gfp* showed improved antioxidant status in contrast to group 4 rats receiving native probiotic *E. coli* CFR 16 which can be explained on the basis of previous findings, demonstrating role of *vgb* gene in improving oxidative status. Oxidative stress is usually implicated in the development and progression of cancer as well as in genesis of other diseases.

Accumulating evidences have supported the generation of ROS in various carcinogenic processes **(Manju et al., 2005)**. DMH also requires metabolic activation through a series of oxidative reactions taking place in the liver to become active electrophilic carcinogen **(Devasena et al., 2006)**. Our findings are in agreement with

higher LPO in the liver of group 2 which could be attributed to DMH-induced oxidative stress and production of reactive oxygen metabolites (ROMs). Colon lipid peroxidation also showed similar pattern as found in liver tissue. PQQ produced from *pqq* gene cluster enhanced antioxidant property of probiotic *E. coli* CFR 16 in comparison to probiotic *E. coli* CFR 16::*vgb-gfp*. Moreover, colon length of groups 3, 7 and 8 showed more protection in comparison to groups 4, 5, 6 and 7 against DMH. In addition, colon length of group 8 was significantly higher in comparison to vitamin C group, suggesting better protection by PQQ producing probiotic *E. coli* which could be attributed to the probiotic effects on the epithelial cell and sustained release of PQQ as compared to the oral dose of antioxidants.

ROS can alter the cellular antioxidant defence system by acting as initiators, promoters and pro-carcinogen activators **(Sengottuvelan et al., 2006)**. Protection from ROS is provided by defence mechanisms of our body which plays a very crucial role. Our current results support previous findings demonstrating declined activities of SOD and CAT in the DMH-treated rats. Present study shows that probiotic *E. coli* CFR 16 may also protect against oxidative stress to certain extent which can be increased further upon incorporation of *vgb* gene. However, most efficient antioxidant property can be achieved by introducing *pqq* gene cluster. In this study, vitamin C was used as a standard antioxidant at concentration almost thousand times more than orally fed PQQ. Orally fed PQQ showed restoration of antioxidant enzyme activity to almost the same extent as shown by vitamin C. Probiotic *E. coli* CFR 16::*vgb-gfp* (*pqq*) fed rats showed significant restoration of SOD activity, with more protection in histological sections in comparison to vitamin C-fed rats. Although orally fed PQQ and PQQ secreted by probiotic *E. coli* were able to restore antioxidant enzyme activity to similar extent, the histological sections showed better in maintaining the tissue architecture in probiotic *E. coli* CFR 16:: *vgb-gfp* (*pqq*) fed rats in comparison to rats fed with PQQ orally.

Stressors including UV radiation, microbes, allergens and various pollutants such as increased ozone, cigarette smoke and polycyclic aromatic hydrocarbons are present ubiquitously in environment which when exposed to body, amplify the generation of reactive oxygen species (ROS) **(Bouayed et al., 2010)**. Endogenous and exogenous antioxidant defence system of body includes various enzymes and

principle dietary antioxidants from fruits, vegetables and grains. DMH is known to be environmental pollutant and can generate systemic oxidative stress leading to alteration in brain neurotransmitter status **(Genc et al., 2012)**. To prevent oxidative damage, natural antioxidants having wide range of biochemical activities including inhibition of ROS generation, scavenging of free radicals and alteration of antioxidant potential has been used **(Finkel et al., 2000)**. Additionally, probiotics are also opted as antioxidants and gram positive probiotics including *Lactobacillus* and *Bifidobacterium* species have been widely used **(Collado et al., 2012)**. In current study, we also demonstrated the potential of PQQ producing probiotic on systemic antioxidant status and neurotransmitter status in rat brain. Our results are in accordance with studies which demonstrated, PQQ supplementation improves antioxidant status of host **(Misra et al., 2012)**. LPO levels and antioxidant enzyme activities also exhibited significant recovery in orally fed PQQ, PQQ producing probiotic *E. coli* and Vitamin-C fed rat groups which are consistent with previously reported data **(Miyauchi et al., 1999)**. As stated earlier systemic oxidative stress can serve as pathological consequence of neurological disorders which may be due to alteration in neurotransmitter status **(Uttara et al., 2009)**.

Defining symptom of major depression is widely recognized as depressed mood, a common ailment in today's world **(Nutt et al., 2007)**. Functional neuroimaging studies have associated depressed mood and sadness with abnormal neuronal activity in the medial prefrontal cortex, including the anterior cingulate and orbitofrontal cortex of brain. These brain regions receive innervation from serotonergic (midbrain raphe), noradrenergic (locus coeruleus) and dopaminergic (ventral tegmental, VTA) pathways and in turn are involved in regulatory function of mood. Low levels of NE, 5-HT and DA may be associated with low mood. Antidepressants involved in enhancing levels of these monoamine neurotransmitters have been shown to improve depressed mood and sadness. Therefore, it was interesting to find the effect of DMH on the brain neurotransmitter status.

Quinones/semi-quinones generated by superoxide radical can deplete reduced glutathione (GSH) and bind to protein SH groups **(Spencer et al., 1998)**. Oxidation can be catalyzed by transition metal ions, but if excess free radicals are present, they can react with norepinephrine, and serotonin to initiate their oxidation, which then

continues with production of more ROS **(Wrona et al., 1998)**. The restoration of biogenic amines in PQQ administered group can be correlated with the fact that being a powerful antioxidant, PQQ enhances the activities of the antioxidant enzymes and may protect the brain neurotransmitters from auto-oxidation. Dopamine levels did not show significant changes in all the groups. However, epinephrine level showed significant increase in DMH treated rats which were reduced near to normal levels in probiotic and Vitamin-C treated group.

Most probiotics are Gram positive among which lactic acid-producing bacteria (LAB) are predominant, and some of them have good antioxidant activity **(Wolvers et al., 2010; Amaretti et al., 2013)**. LAB effects documented in humans are immune stimulation, diarrhea reduction/prevention and reduced lactose intolerance. They are also known to synthesize vitamins, improve mineral and nutrient absorption, degrade anti-nutritional factors and/or modulate GI physiology. However, they do not possess the ability to synthesize PQQ. On the other hand, only few Gram-negative probiotics are commercially available, i.e. *E. coli* Nissle 1917 (EcN) **(Sonnenborn et al., 2009)**. EcN carries several fitness factors, e.g. microcin production, mutation in lipopolysaccharide (LPS) biosynthesis gene. Bacterial–epithelial cross talk between EcN and the intestinal epithelial cells leads to strengthening of the epithelial barrier and the curing of 'leaky gut' phenomena. In addition, EcN has also been shown to induce the development of the gut immune system in animal models and human newborns, promotes colonic motility and is effective in most of gut disorders. Antimicrobial potential of *E. coli* CFR 16 could also be effective in preventing enteropathogen infections **(Kumar et al., 2009)**. However, similar to other *E. coli* strains, it does not possess PQQ biosynthesis genes, even though they encode glucose dehydrogenase apo-protein **(Matsushita et al., 1997)**.

In addition to antioxidant potential, PQQ has been reported to improve energy utilization and reproductive performance by modulating gene expression patterns and transcriptional networks involved in cellular stress (e.g. thioredoxin), mitochondriogenesis, cell signalling (JAK/ STAT and MAPK pathways) and transport. In addition, in RNA transcript expression profiling revealed, PQQ repletion altered 847 of total transcripts and reversed the changes in transcript expression caused by PQQ deficiency. These effects of PQQ will be explored in future studies.

Our current study integrates beneficial properties of PQQ, Vhb and probiotic *E. coli* CFR 16 for enhancing the efficacy of probiotic *E. coli* for ameliorating oxidative stress.

2.4 Conclusion

PQQ produced by probiotic *E. coli* CFR 16::*vgb-gfp* (*pqq*) prevented oxidative stress in colonic and liver tissue induced by administration of DMH. This suggests that probiotic *E. coli* CFR 16::*vgb-gfp* (*pqq*) possesses high antioxidant capacity combined with the positive effects of PQQ, Vhb and *E. coli* CFR 16 implicated in the prevention of oxidative stress related diseases and neuro-modulatory effects.