Chapter 2

Evaluating effect of PQQ secreting *Escherichia coli* strains on ethanol mediated oxidative damage and dyslipidemia in rats

2.1 Introduction

Consumption of alcohol is a major factor contributing to various metabolic disorders and is chiefly involved in oxidative liver damage attributing to alcoholic liver disease (Gao and Bataller, 2011). In adults, ethanol (EtOH) is primarily metabolized in liver by alcohol dehydrogenase to acetaldehyde which is further oxidized to acetate by acetaldehyde dehydrogenase. Acetate then enters mitochondria in lipid biosynthetic pathway (Lundquist et al., 1962). Elevated levels of plasma triglycerides, total cholesterol, and high-density lipoprotein (HDL) cholesterol have been found to be associated with alcoholic patients (Crouse and Grundy, 1984). In addition, microsomal EtOH-oxidizing system (MEOS) and catalase in peroxisomes are also involved in oxidation of EtOH to acetaldehyde (Lieber, 1999).

EtOH, apart from providing high calories leading to dyslipidemia and obesity (Baraona and Lieber, 1979), is a very potent pro-oxidant causing cellular oxidative damage (Koch et al., 2004). Most of its pro-oxidant effect is attributed to acetaldehyde (Niemela, 2001). Acetaldehyde forms adducts with DNA leading to their destabilization (Brooks and Theruvathu, 2005). Free radicals generated by acetaldehyde causes lipid peroxidation (French et al., 1993), and protein carbonylation and cytoskeletal damage (Banan et al., 1999). Acetaldehyde induces barrier disruption by protein phosphatase 2A-dependent mechanism in Caco-2 cells, which is considered as one of the major factors contributing to alcoholic liver disease (Dunagan et al., 2012). Pyrroloquinoline quinone (PQQ) reduced acetaldehyde levels in blood and liver after EtOH ingestion in rats (Hobara et al., 1988). Moreover, probiotics such as *Lactobacillus* GG have been shown to prevent EtOH-induced intestinal and liver damage through induction of hypoxia-inducible factor and intestinal trefoil (Wang et al., 2011).

PQQ, a bacterial cofactor for variety of dehydrogenases and oxidoreductases, is considered as one of the most efficient water soluble, heat stable antioxidant having redox catalytic cycle of 20,000, which considerably higher than quercetin, epicatechin, and vitamin C (Rucker et al., 2009). Cell culture studies have demonstrated that PQQ is capable of

preventing neuronal damage caused by methyl mercury and glutamate-induced apoptosis and enhances brain regenerative capacity. It is a vital nutrient required for normal growth and development in rodent models. A recent study shows that PQQ inhibits oxygen/glucose deprivation-induced apoptosis in cardiomyocytes (Xu et al., 2014). PQQ and its derivatives have been detected in human and rat organs in trace amounts with the highest in human milk ranging from nanomolar to micromolar concentration (Kumazawa et al., 1992; Mitchell et al., 1999). Bauerly and colleagues (2011) reported elevated plasma triglyceride levels in rats fed with PQQ deficient diet, which decreases significantly (2-fold) upon intraperitorial administration of PQQ. Increased mitochondrial biogenesis was also found in rats fed with PQQ supplemented diet.

Escherichia coli Nissle 1917 (EcN; Mutaflor; Ardeypharm GmbH, Loerfeldstrabe 20, Herdecke [Germany]) is shown to alleviate oxidative stress in experimental colitis and prevents inflammatory bowel disease (Grabig et al., 2006; Schultz, 2008). Although, many Gram-negative bacteria synthesize PQQ, *E. coli* strains appear to have lost this property (Matsushita et al., 1997). Incorporation of *pqq* gene cluster enables *E. coli* to secrete high amounts of PQQ (Yang et al., 2010). *Vitreoscilla* hemoglobin (VHb) is known to improve the growth of bacteria under oxygen limited conditions (Kallio et al., 1994). As intestine has less oxygen tension, VHb could be beneficial for bacterial colonization in the gut. The present study demonstrates the effect of PQQ-secreting *E. coli* on the oxidative damage and hyperlipidemia caused by chronic EtOH ingestion in rats.

2.2 Methods and materials

2.2.1 Bacterial Strains and Culture Conditions

EcN was obtained as a generous gift from Dr. Rer. Nat. Ulrich Sonnenborn, Ardeypharm GmbH, Loerfeldstrabe 20, Herdecke (Germany). EcN and its modifications are summarized in Table 3.1. To generate EcN strain with genomic integration of *vgb* and *gfp* genes (EcN-2), both genes were first cloned in pGRG36 integration vector and then transformation and integration were performed as per standard protocol (McKenzie and Craig, 2006). Confirmation of integration was done by polymerase chain reaction (PCR). All strains were grown in Luria broth at 37 °C and cultures were washed with sterile normal saline and suspended in it before feeding to rats. PQQ quantification was done using supernatant of 24-hour grown culture in M9 minimal medium containing glucose as sole carbon source. PQQ extracted from *E. asburiae* PSI3 was used for feeding purpose after quantification.

2.2.2 Plasmids and constructs

pBBR1-MCS2 plasmid harbouring *Pseudomonas fluorescens* B16 *pqq* gene cluster was used for the transformation of EcN.

Table 2.1 Plasmids and Bacterial strains

Strains/ Plasmids	Characteristics	Reference
Plasmids		
pBBR1 MCS 2	Broad host range plasmid,*lacI*$^{-}$, multiple cloning site in *lacZ* gene; Kan^{r}	Kovach et al., 1995
pBBR1 MCS 2-*pqq*	pBBR1MCS2 harboring *Pseudomonas fluorescens* B16 *pqq* gene cluster (13.4 Kb)	Pandey et al., 2014
Strains		
EcN	*Escherichia coli* Nissle 1917 (EcN) (Probiotic strain)	Sonnenborn and Schulze, 2009
EcN-2	EcN strain with genomic integration of *vgb* and *gfp* genes	This study
EcN-3	EcN-2 strain harboring pBBR1 MCS2 plasmid	This study

EcN-4	EcN-2 strain harboring pBBR1 MCS2-*pqq* plasmid	This study
Enterobacter asburiae PSI3	PQQ producing, phosphate solublizing bacteria isolated from pigeon pea rhizosphere	Gyaneshwar et al., 1999
EcCFR16	*Escherichia coli* CFR 16 strain (Probiotic strain)	Kumar et al., 2009
EcCFR16:: *vgb-gfp*	EcCFR16 with genomic integration of *vgb* and *gfp* genes	Pandey et al., 2014
EcCFR16:: *vgb-gfp* (VC)	EcCFR16:: *vgb-gfp* strain harboring pBBR1 MCS2 plasmid	Pandey et al., 2014
EcCFR16:: *vgb-gfp* (*pqq*)	EcCFR16:: *vgb-gfp* strain harboring pBBR1 MCS2-*pqq* plasmid	Pandey et al., 2014

2.2.3 Animals

Charles Foster male albino rats weighing 180 to 220 g and 10 to 12 weeks of age were selected for the present study. They were maintained in controlled conditions (temperature: 25 ± 1°C; relative humidity: 45.5 %; photoperiod cycle: 12 h light and 12 h dark) with free access to food and water as per recommendations from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), an animal ethical committee of the institute (The Maharaja Sayajirao University of Baroda, India, Reg. No. 938/A/06/CPCSEA). Animals received diet containing 64 % starch, 20 % casein, 0.7 % methionine, 5 % groundnut oil, 9.7 % wheat bran, 3.5 % salt mixture, and tap water ad libitum. The salt mix in a kg contained $MgSO_4$. $7H_2O$, 30.5 g; NaCl, 65.2 g; KCl, 105.7 g; KH_2PO_4, 200.2 g; $MgCO_3$, 3.65 g; $Mg(OH)_2$. $3H_2O$, 38.8 g; $FeC_6H_5O_7$. $5H_2O$, 40.0 g; $CaCO_3$, 512.4 g; KI, 0.8 g; NaF, 0.9 g; $CuSO_4$. $5H_2O$, 1.4 g; $MnSO_4$, 0.4, and $CONH_3$, 0.05 g; and water ad libitum. Two-tenths milliliter of vitamin mixture (the vitamin mix in a kg contained vitamin A concentrate I.P., 2,500 I.U.; vitamin D3 cholecalciferol, 200 I.U.; thiamine hydrochloride, 0.5 mg; riboflavin, 0.5 mg; pyridoxin, 0.5 mg; sodium pantothenate, 1.5 mg; nicotinamide, 5 mg; ascorbic acid, 25 mg multivitamin tablets; Piramal Healthcare Ltd., Mumbai, India) was added per kg feed. The diet contained 25 ± 4 mg of PQQ per kg.

2.2.4 Designing of experiments

Firstly, standard dose and duration of ethanol which created systemic oxidative stress and dyslipidemia, correlating with onset conditions of alcoholic liver disease, was optimized. Rats were given daily ethanol dose of 1.5 g/Kg body weight and 3 g/Kg body weight. Plasma samples were analysed for oxidative stress markers and lipid profile at week 5 and 10. Rats were gradually adapted for ethanol dose (Figure 2.1).

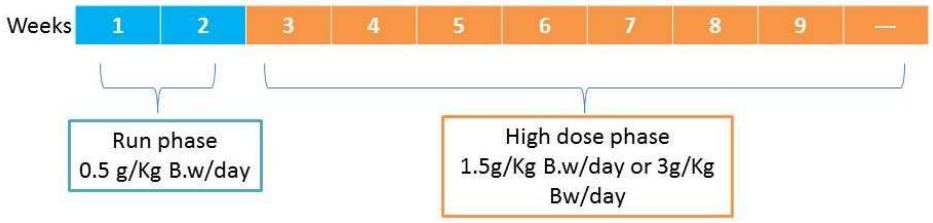

Figure 2.1: A schematic representation of protocol followed for chronic ethanol administration in rats.

For chronic study, rats were randomly divided into 8 different groups receiving different treatments as described in Figure 2.2.All rats received normal pellet diet with full access to drinking water. All rats were first treated with streptomycin (5 g/l) for 24 h. Then probiotic treatment was given for consecutive 2 days to respective groups, and fecal samples were analysed on 15th day for confirmation of colonization by visualizing *gfp* expressing colonies. Once colonization was confirmed, EtOH treatment was started. EtOH (3 g/ kg body weight/d) was given intragastrically twice a day for 10 weeks. EtOH-treated groups were subjected to a gradual adoption period of 2 weeks where EtOH was given in increasing concentration starting from 5 % v/v reaching up to 25 % v/v, till the end of second week. From the third week, 25 % v/v EtOH was continued till the end of the experiment. Probiotic culture (10^8 CFU), PQQ external (10 mg/kg body weight), and vitamin C (350 mg/kg body weight) were fed intragastrically once a week.

For acute experiment, rats were divided into 6 groups: Untreated, EtOH, PQQ (Ext), EcN-3 (EtOH + probiotic EcN-3), EcN-4 (EtOH + probiotic EcN-4), and vitamin C (EtOH + vitamin C). EtOH (30 % v/v) was given intragastrically. Probiotic treatment (10^8 colony-forming units [CFU]/rat for 3

consecutive days) following streptomycin wash (5 g/l for 24 h) was given orally dissolved in sterile normal saline. The colonization of these probiotics was confirmed after 15 days of first treatment by fecal count and then EtOH (5 g/kg body weight) was ingested orally External PQQ (10 mg/kg body weight) and vitamin C (350 mg/ kg body weight) were given 2 h before EtOH treatment. After 18 h of EtOH treatment, rats were sacrificed, and samples were collected for analysis.

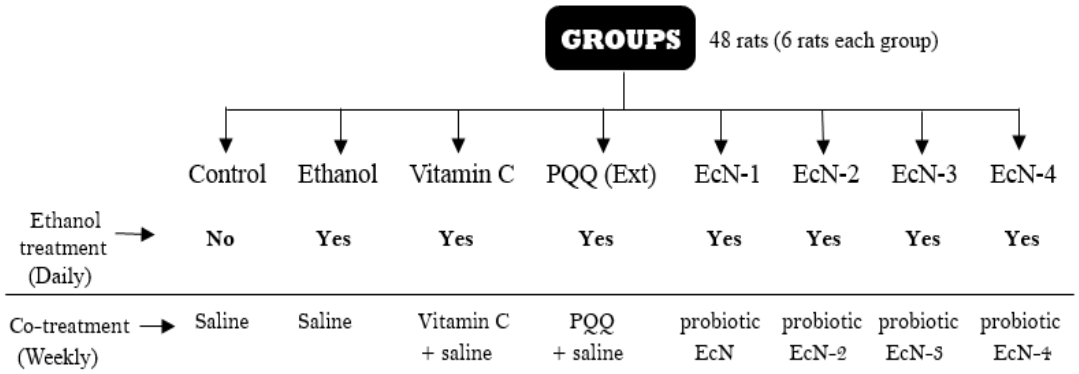

Figure 2.2 Experimental groups and treatments for evaluating their effect against chronic ethanol ingestion. Vitamin C and PQQ were dissolved in water whereas probiotic strains were suspended in normal saline before treatment.

2.2.5 Characterization of PQQ secreting EcN (EcN-4)

Probiotic strain EcN-2 was transformed with pBBR1-MCS2 vector harbouring *pqq* gene cluster (pBBR1 MCS2-*pqq*) by $CaCl_2$ method (Sambrook et al., 2001). Functionality of the transformant was confirmed by growing in Tris buffered medium with methyl orange as pH indicator (Gyaneshwar et al., 1999). Red coloration indicated pH drop.

2.2.6 PQQ extraction and quantification

PQQ was extracted from *E. asburiae* PSI3 and EcN strains as described by Suzuki et al. (1990) and used by Rajpurohit et al. (2008). Overnight grown cells in M9 minimal medium containing glucose were harvested and the supernatant was used for PQQ extraction. Culture supernatant was incubated with 50 % acetonitrile at 65 ^{0}C for 2 h followed by centrifugation at 15,000 g for 10 minutes. The clear supernatant obtained was

collected and dried using concentrator in vacuum. The residues were dissolved in 50 % *n*-butanol (1 mg/ml) and were incubated at 50 ^{0}C till it dries. Finally the residues obtained were dissolved in water and filtered with 0.2 micron filter. Quantification was done fluorimetrically as described by Suzuki et al. (1990) using Hitachi fluorescence spectrophotometer with excitation 375 nm and emission 465 nm (uncorrected). Standard plot for area under curve was drawn using 6 different concentration of standard PQQ ranging from 0.2 µM to 20 µM. Liver tissue was homogenized in phosphate buffered saline (20 % homogenate) followed by centrifugation at 10000 g for 20 minutes. To the supernatant 50 % acetonitrile was added for PQQ extraction and quantification as described above. Colonic contents were collected after sacrificing rats and immediately stored at -80 ^{0}C till use. Samples were thawed and vigorously mixed and homogenized with phosphate buffered saline (10 % homogenate) and centrifuged at 10000 g for 20 minutes. To the supernatant, 50 % acetonitrile was added for PQQ extraction and quantification as described above. Recovery of PQQ in samples (Fecal and Liver with added PQQ) was consistently greater than 82 %.

2.2.7 Preparation of cell lysate and tissue homogenate

Blood collected in EDTA tubes was centrifuged at 1500 g for 10 minutes to separate plasma and pack cell volume (PCV). Plasma was transferred in different tubes and kept at -80 ^{0}C till use. PCV was washed twice with normal saline and centrifuged at 1500 g for 10 minutes. PCV lysate was prepared with ice cold water and was used for enzyme assays. Liver, colon and kidney removed immediately after sacrificing rats, were washed with ice cold phosphate buffer saline and kept at -80 ^{0}C till homogenization in an appropriate buffer. For GSH estimation from liver tissues, homogenate were prepared in ice cold 5 % TCA. And for rest of the assays, tissues were homogenized in ice cold phosphate buffered saline.

2.2.8 Biochemical assays

Measurement of **Superoxide Dismutase (SOD**) activity was carried out by using pyrogallol method (Marklund and Marklund, 1974). **Catalase** activity was measured by disappearance of hydrogen peroxide at 240 nm

(Beers and Sizer, 1952). One unit of enzyme is defined as 1 µ mol of hydrogen peroxide consumed per minute, and specific activity is reported here in units/ milligram protein. **Reduced Glutathione (GSH)** was estimated as described by Beutler et al. (1969). Briefly, tissues homogenate in 5 % TCA was centrifuged at 12000 g for 20 minutes (2-4 ^{0}C) and supernatant was directly used for estimation. **Lipid peroxidation** was measured by estimating levels of MDA in various tissues as described by Buege and Aust (1978).

2.2.9 Histopathological changes

Liver tissue was fixed with 10 % buffered formalin. Histological sections were stained with hematoxylin and eosin. Pathologist unaware with experimental codes was consulted for analysis.

2.2.10 Serum Glutamate Pyruvate Transferase (SGPT) activity, total bilirubin and blood lipid estimation.

SGPT activity was measured using kit as per manufacturer protocol (Coral clinical system, India). Total bilirubin, triglyceride, HDL-cholesterol and total cholesterol content in blood plasma were measured using kit as per manufacturer protocol (Beacon Diagnostics Pvt. Ltd., Navsari, Gujarat, India).

2.2.11 mRNA expression and qRT-PCR of hepatic lipid metabolizing genes

RNA was extracted with Trizol (Invitrogen) and cDNAs were generated from 1 µg total RNA (Reverse Transcription Kit, Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Primers selected for Fatty acid synthase and Acyl Co-enzyme A gene were ACCTCATCACTAGAAGCCACCAG (forward) and GTGGTA CTTG GCCTTGGGTTTA (Reverse), and CCCAAGACCCA AGAGTTCATTC (Forward) and TCACGGATAGGGACAACAAAGG (Reverse), respectively. PCR was performed by ABI QuantStudio™ 12K flex Real Time PCR system coupled with SYBR Green technology (Applied Biosystems) and following cycling parameters. The linearity of the dissociation curve was analyzed using the software provided with the thermocycler (QuantStudio™). Each sample

was analysed in duplicate. The mean cycle time of the linear part of the curve was designated *Ct*.

2.2.12 Estimation of colonic SCFAs

Colonic content was collected immediately after dissection of rats and stored at -80 ^{0}C till use. Samples were taken from -80 ^{0}C and suspended in sterile deionized water containing 0.015 M H_2SO_4. Sample preparation and detection of SCFAs were done as described by Chen and Lifachitz (1989), using Shimadzu HPLC system with C-18 column.

2.2.13 Statistical analysis

The statistical significance of the values has been determined by one way ANOVA using GraphPad Prism Version 5.0 (GraphPad software, Inc.). The results were considered significant at $p \leq 0.05$.

2.3 Results

2.3.1 Standardization of optimal ethanol dose and duration to created ethanol induced model of systemic oxidative stress and dyslipidemia

Change in body weight

Rats treated with 3 g/kg body weight ethanol showed more significant reduction in body weight gain as compared to control and those treated with 1.5 g/kg body weight ethanol (**Figure 2.3**).

Antioxidant status

Rats treated with high dose of ethanol (3 g/kg bw) showed more oxidative stress as measured by plasma MDA, hepatic MDA and hepatic GSH (**Figure 2.3**). At week 5 plasma MDA levels were significantly elevated in both the ethanol treated groups. However, rats receiving ethanol (3 g/kg bw) showed more elevation as compared to rats receiving ethanol (1.5 g/kg bw). At week 10, rats receiving ethanol (3 g/kg bw), showed remarkable further increase in MDA levels as compared to control and ethanol (1.5 g/kg bw) groups. Also, we could see significant elevation in Hepatic MDA levels in rats receiving high dose of ethanol as compared to other groups. Moreover, these rats also showed more reduction in hepatic GSH as compared to other groups.

Liver function

Rats treated with high dose of ethanol (3 g/kg body weight) showed remarkable increase in SGPT activity at week 5 **(Figure 2.3)**. However, low dose of ethanol (1.5 g/kg bw) did not alter the activity at week 5. Moreover, at week 10 SGPT activity was significantly increased in both the ethanol treated groups, with highest in rats treated with ethanol (3 g/kg bw).

Lipid profile

Blood lipid profile measured at week 5 of ethanol treatment did not show any significant increase in triglycerides, total cholesterol and HDL cholesterol levels in rats treated with low dose ethanol (1.5 g/kg bw). Also, rats treated with high dose ethanol (3 g/kg bw) shoed only marginal increase

in blood triglycerides whereas total cholesterol and HDL cholesterol remained significantly unchanged. At week 10, rats receiving low dose ethanol (1.5 g/kg bw) showed significant increase in plasma triglycerides, whereas there were no significant increase in total cholesterol and HDL cholesterol levels. However, rats receiving high dose of ethanol (3 g/kg bw) exhibited significant increase in plasma triglycerides, total cholesterol and HDL cholesterol levels as compared to other two groups **(Figure 2.3)**.

Hepatic lipid profile measured at the end of experiment (week 10), showed that rats receiving low dose ethanol had no change in hepatic total cholesterol although there was significant increase in hepatic triglycerides. However, rats receiving high dose ethanol had significantly high level of hepatic total cholesterol and triglycerides as compared to two other groups **(Figure 2.3)**.

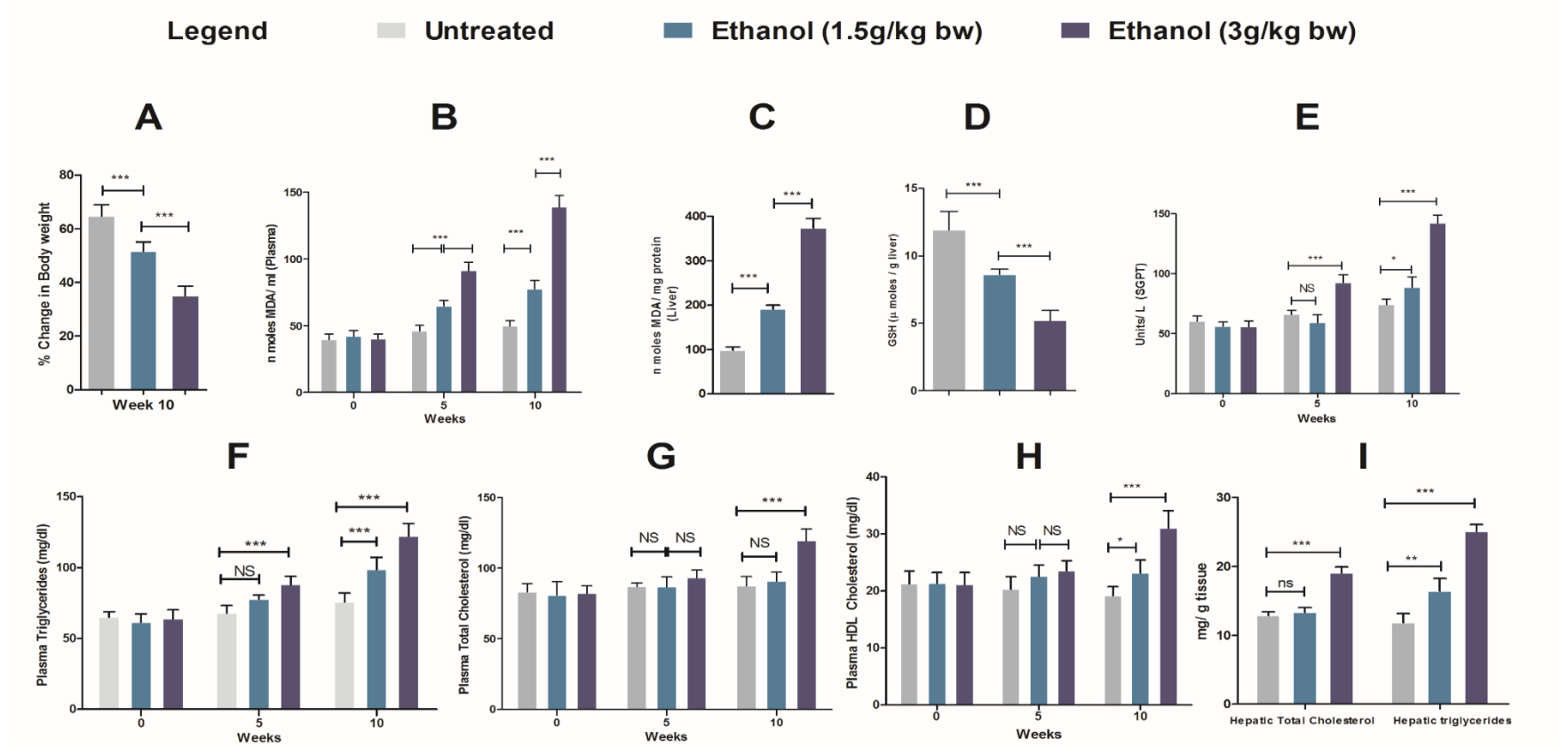

Figure 2.3 Standardization of optimal ethanol dose and duration. All values are represented as mean ±SEM of 6 animals each group. ***p≤ 0.001, **p≤ 0.01, *p≤ 0.05. **(A)** Percentage change in body weight, **(B)** Lipid peroxidation in plasma, **(C)** Lipid peroxidation in liver, **(D)** Reduced glutathione in liver, **(E)** SGPT activity, **(F)** Triglyceride in blood, **(G)** Total cholesterol in blood, **(H)** HDL cholesterol in blood and **(I)** Hepatic triglyceride and total cholesterol.

2.3.2 Transformation of probiotic *E. coli* strains with plasmid harbouring *pqq* gene cluster

pBBR1 MCS2 vector harbouring 13.3 Kb *pqq* gene cluster from *P. fluorescens* B16 was used for transforming probiotic *E. coli* strains. Firstly, vector was digested with appropriate restriction enzyme to confirm the presence of *pqq* gene cluster followed by transformation in probiotic *E. coli* Nissle and CFR16 strains. Digestion pattern revealed 13.3 Kb insert release of *pqq* genes and 5.1 Kb vector backbone confirming the clone **(Figure 2.4)**.

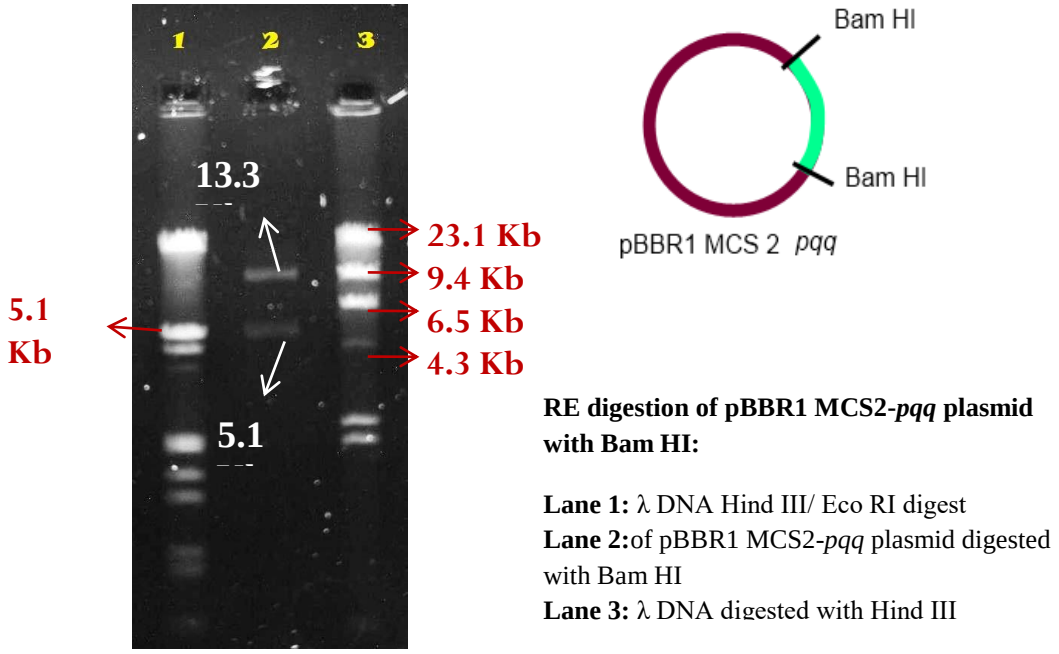

Figure 2.4 Confirmation of pBBR1 MCS2-pqq plasmid by Restriction Enzyme digestion.

After confirmation probiotic *E. coli* strains Nissle 1917 and CFR16 were transformed with pBBR1 MCS2 vectors with and without *pqq* genes. The vector contains kanamycin resistance gene, which was used for selection. Strains harbouring plasmid showed growth (**Figure 2.5**).

Probiotic *E. coli* strains (*E. coli* Nissle 1917 and *E. coli* CFR16) harbouring *pqq* gene cluster were grown in M9 minimal medium and PQQ in

the supernatant was quantified after 72 h at 37 ^{0}C at 200 rpm **Table 2.2)**. Also, transformants were confirmed by growth and acidification on Tris buffered medium containing methyl red as pH indicator.

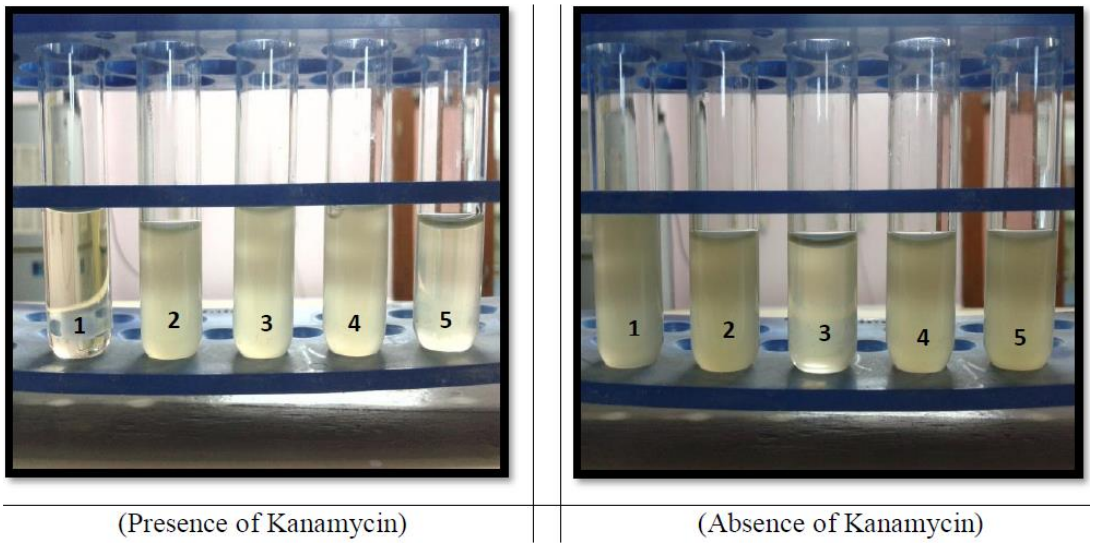

Figure 2.5*E. coli* strains grown on Luria broth with and without kanamycin. EcN wild type strain (1), EcN-3 (2), EcN-4 (3), EcCFR16::*vgb-gfp* (VC) (4) and EcCFR16:: *vgb-gfp* (*pqq*) (5).

Table 2.2 PQQ secreting in supernatant by probiotic *E. coli* strains

Strains	EcN-4	EcCFR16:: *vgb-gfp* (*pqq*)
PQQ in supernatant	1.68 ± 0.1 µg/ ml culture (n=3)	1.61 ± 0.08 µg/ ml culture (n=3)

2.3.3 Effect of EcN-4 secreting PQQ against acute ethanol induced oxidative stress

EcN-3 and EcN-4were found to be 10^{7-8} CFU per g fecal matter at the time of acute ethanol treatment. Acute high ethanol ingestion increased lipid peroxidation levels in serum and liver homogenate, and decreased reduced Glutathione (GSH) levels in liver (**Figure 2.6**). Significant reduction in blood and hepatic MDA levels were found in rats pre-treated with PQQ(Ext), EcN-4and Vitamin C along with elevation of GSH levels up to normal when compared to only ethanol treated rats. EcN-4 and Vitamin C treatment

showed similar effect, but in comparison, PQQ (Ext) treatment had much pronounced effect.

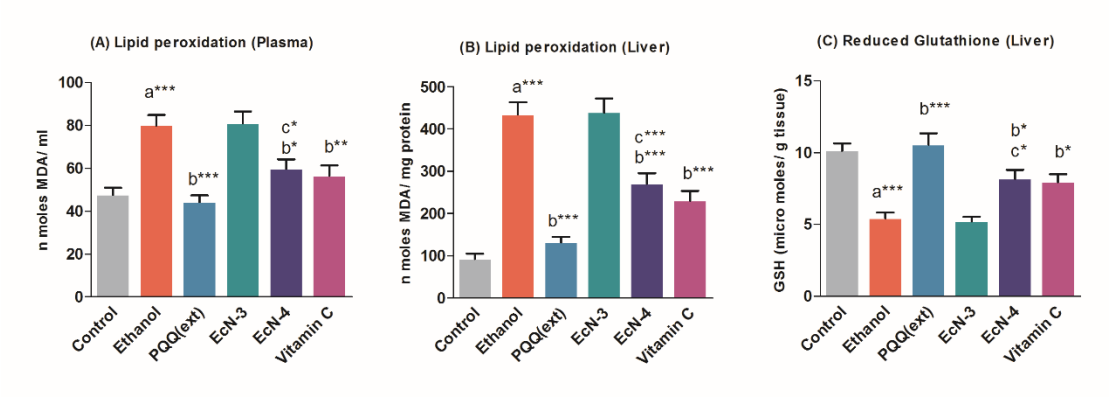

Figure 2.6 Effect of PQQ, EcN-4 and Vitamin C pre-treatments on oxidative damage and antioxidant status measured as MDA levels in Plasma (A) and Liver (B), and reduced hepatic glutathione levels (C) in acute ethanol fed rats. Values are mean ± SEM (n=6 each group). a***p≤ 0.001 compared to untreated group. b*p≤ 0.05, b**p≤ 0.01 and b***p≤ 0.001 compared to ethanol group. c*p≤ 0.05 and c***p≤ 0.001 compared to EcN-3 group.

2.3.4 Effect of EcN-4 secreting PQQ on antioxidant status in chronic ethanol treated rats

Effect on liver antioxidant status and liver function parameters

Lipid peroxidation measured in the form of MDA was found to be significantly higher in ethanol treated rats as compared to control (**Figure 2.7A**). Co-treatment of EcN-4 significantly reduced MDA levels to normal. Whereas, rats co-treated with vitamin C and PQQ (Ext) did not show any reduction in MDA levels. Reduced Glutathione (GSH) levels in ethanol treated rats were lowered up to 50 % as in control rats (**Figure 2.7B**). EcN-4 co-treatment effectively enhanced GSH up to normal level. No restoration of GSH levels in other experimental groups was found. Similarly, SOD and Catalase activities which were significantly reduced in ethanol treated rats were found restored in rats co-treated with EcN-4 (**Figure 2.7C and D**). Any restoration in the activities of these enzymes in other experimental groups was not observed. As a result of chronic ethanol treatment, SGPT activity and total plasma bilirubin were found elevated by more than 2 fold and 1.5 fold, respectively (**Figure 2.7 E and F**). EcN-4 co-treatment decreased SGPT activity as well as

total plasma bilirubin to near normal levels, indicating proper functioning of the liver. Rats co-treated with Vitamin C and PQQ (Ext) did not show any significant reduction when compared to only ethanol treated rats.

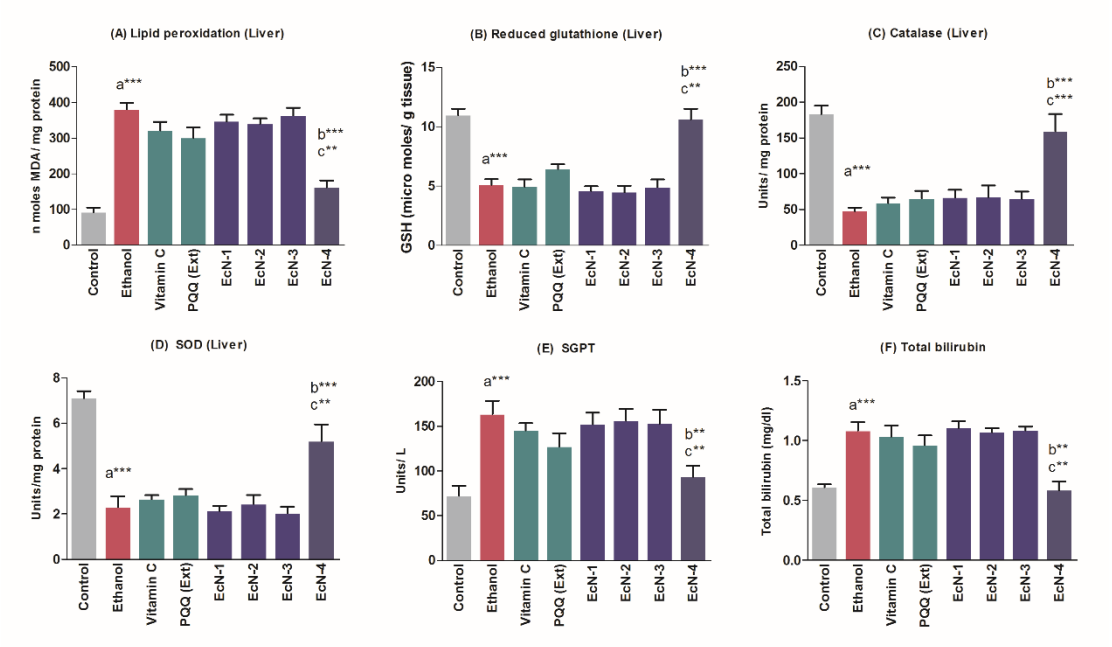

Figure 2.7 Effect of EcN-4 on hepatic oxidative damage, antioxidant status and liver function parameters in chronic ethanol fed rats. Oxidative damage as measured by hepatic MDA levels (A). Antioxidant status measured by reduced glutathione levels (B), catalase (C) and SOD activities (D) in hepatic tissue. SGPT (E) and total bilirubin (F) are markers of liver function measured in serum. Values are mean ± SEM (n=6 each group). a***p≤ 0.001 compared to untreated group. b***p≤ 0.001 and b**p≤ 0.01compared to ethanol group. c**p≤ 0.01 and c***p≤ 0.001 compared to PQQ (Ext) group.

Changes in liver histology

To evaluate the effect of PQQ secreting EcN (EcN-4) on morphological changes of the liver, histological sections of hepatic tissues were taken and stained with hematoxylin and eosin. The sections were observed under compound microscope at 40X. Chronic ethanol ingestion causes extensive cellular damage as seen by severe steatosis in liver sections of ethanol treated rats (**Figure 2.8**). Rats co-treated with EcN-4 had minimal steatosis which was comparable to control rats. However rats treated with EcN-3 and PQQ (Ext) had no protective effect as visualized through sectioning.

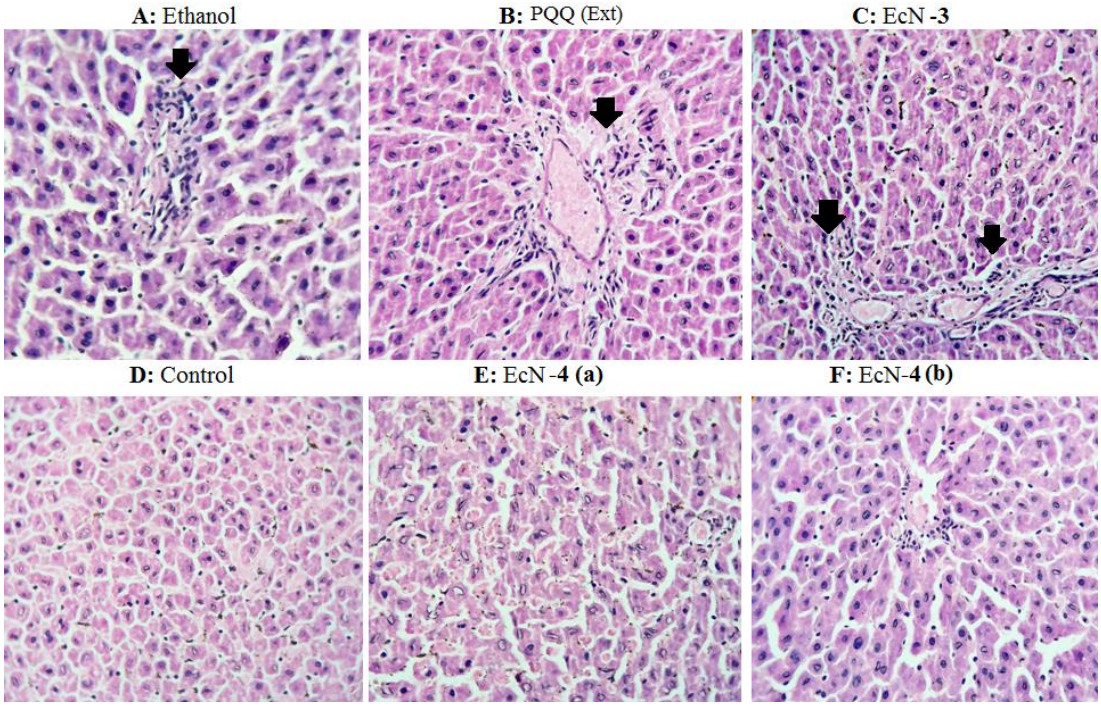

Figure 2.8 Histological sections of liver tissue of the rats treated with chronic ethanol with or without probiotic co-treatments (40X). Hematoxylin Eosin stained. Black arrows indicate sites of severe damage signifying obvious necroinflammation in hepatic sections of rats treated with only ethanol (A), and those co-treated with PQQ (Ext) (B) and EcN-3 (C). Section of control rats shows proper cellular morphology (D). Rats co-treated with EcN-4 along with chronic ethanol shows significant prevention from damage and severe necroinflammation with no apparent morphological difference as compared to control rats (E and F). E and F are two different samples with the same treatment.

Effect on blood, colonic and renal antioxidant status

Plasma lipid peroxidation, which is systemic marker for oxidative damage, was found more than 2.5 fold elevated in chronic ethanol fed rats. Also, SOD and catalase activities in erythrocytes were drastically diminished in these rats (**Figure 2.9 A-C**). Co-treatment with EcN-4 significantly reduced plasma MDA levels up to 70 % and elevated SOD and catalase activities up to near normal levels when compared to ethanol treated rats. Rats co-treated with Vitamin C and PQQ (Ext) had no ameliorative effect on plasma MDA levels and erythrocyte SOD and catalase activities against chronic ethanol treatment. Ethanol treated rats possessed nearly 3 fold elevated MDA levels in colonic tissue (**Figure 2.9 D-E**). Co-treatment of PQQ (Ext), EcN-1, EcN-2 and EcN-3 significantly lowered MDA levels in colonic tissue as compared to

only ethanol treated rats, but were still higher when compared to control rats. However, EcN-4 co-treatment had better effect, with reduction in MDA levels similar up to control rats. Vitamin C co-treatment had no preventive effect in colonic tissue. Reduction in colonic catalase activity observed in ethanol treated rats was found significantly increased by EcN-4 co-treatment. We found no significant elevation in colonic catalase activity in other treated group. Similarly, EcN-4 co-treatment significantly reduced renal MDA levels and elevated renal catalase activity as compared to ethanol treated rats (**Figure 2.9 F-G**). PQQ (Ext) co-treatment resulted in marginal reduction in MDA levels, but we could not find any significant restoration of renal catalase activity in these rats as compared to ethanol treated ones. Vitamin C co-treatment had no effect on MDA levels as well as catalase activity in renal tissue of ethanol treated rats.

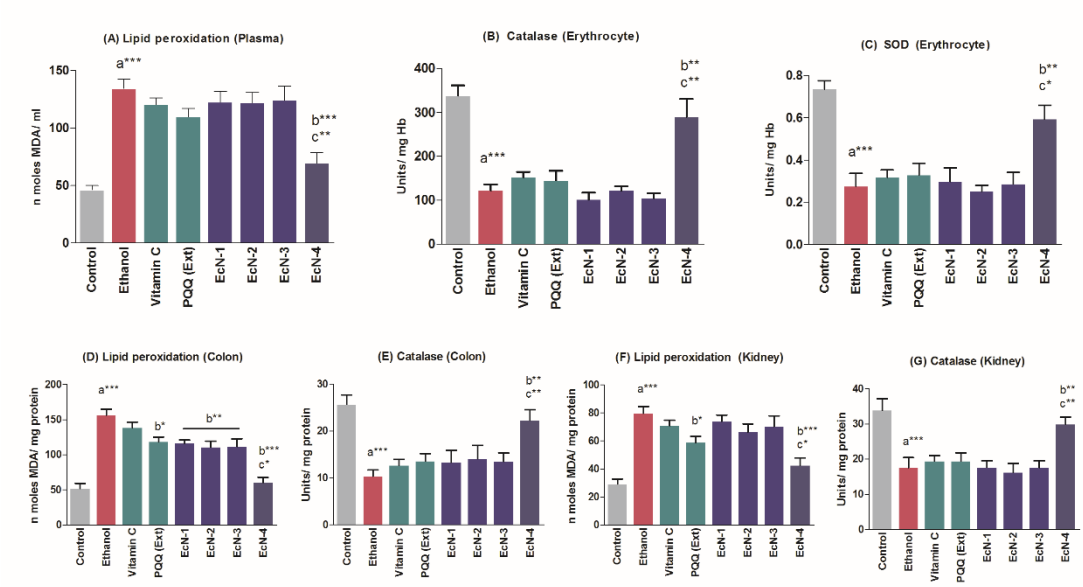

Figure 2.9 Effect of EcN-4 on oxidative damage and antioxidant status in extra hepatic tissues (blood, colonic and kidney) of chronic ethanol fed rats. MDA levels were measured from plasma (A), colon (D) and kidney (F). Erythrocyte catalase (B) and SOD (C) activities were measured to analyse blood antioxidant status. Catalase activity in colonic (E) and renal tissues (G) was measured to analyse antioxidant status in respective tissues. Values are mean ± SEM (n=6 each group). a***p≤ 0.001 compared to untreated group. b*p≤ 0.05, b**p≤ 0.01 and b***p≤ 0.001 compared to ethanol group. c*p≤ 0.05 and c**p≤ 0.01 compared to PQQ (Ext) group.

2.3.5 Effect of EcN-4 secreting PQQ on weight gain, lipid profile and hepatic gene expression in chronic ethanol treated rats

Effect on body weight change, blood and hepatic lipid profile

Chronic ethanol ingestion for 10 weeks resulted in significant reduction in percent body weight gain as compared to control rats (**Table 2.3**). Only EcN-4 co-treated rats showed significant restoration of body weight. Increased blood total cholesterol, triglyceride and HDL cholesterol observed in rats after chronic ethanol treatment, were significantly reduced in the rats co-treated with EcN-4. PQQ (Ext) co-treatment resulted in mild reduction in these plasma lipids as compared to ethanol treatment. However, EcN-4 co-treatment showed better amelioration of blood lipid profile, maintaining to near normal levels. Co-treatment of Vitamin C, EcN-1, EcN-2 and EcN-3 did not show any effect on plasma lipids as compared to only ethanol treatment. Similarly, rats treated with ethanol also had elevated levels of hepatic total cholesterol (1.4 fold) and triglycerides (2 fold) as compared to control. Co-treatment of EcN-4 significantly lowered both total hepatic cholesterol and hepatic triglycerides when compared to only ethanol treated rats. On the other hand, PQQ (Ext) co-treatment had no effect, since the treated rats exhibit no significant restoration of hepatic lipids. Vitamin C, EcN-1, EcN-2 and EcN-3 co-treatment were also found ineffective in ameliorating hyperlipidemia.

Effect on hepatic FAS and ACOx mRNA expression

Nearly, 2.8 fold elevated hepatic FAS mRNA level was observed in ethanol treated rats as compared to control (**Figure 2.10A**). Probiotic EcN-4 co-treatment significantly lowered FAS mRNA expression, as it was found 0.5 fold less compared to only ethanol treated rats. On the other hand, chronic ethanol treatment had no effect on mRNA expression of ACOx (**Figure 2.10B**). However, rats co-treated with EcN-4 had 2.4 fold increased hepatic ACOx mRNA expression as compared to control and ethanol treated rats. Other co-treatments such as PQQ (Ext), EcN-1, EcN-2, EcN-3 and Vitamin C had no significant effect on mRNA expression of hepatic FAS and ACOx genes in chronic ethanol treated rats.

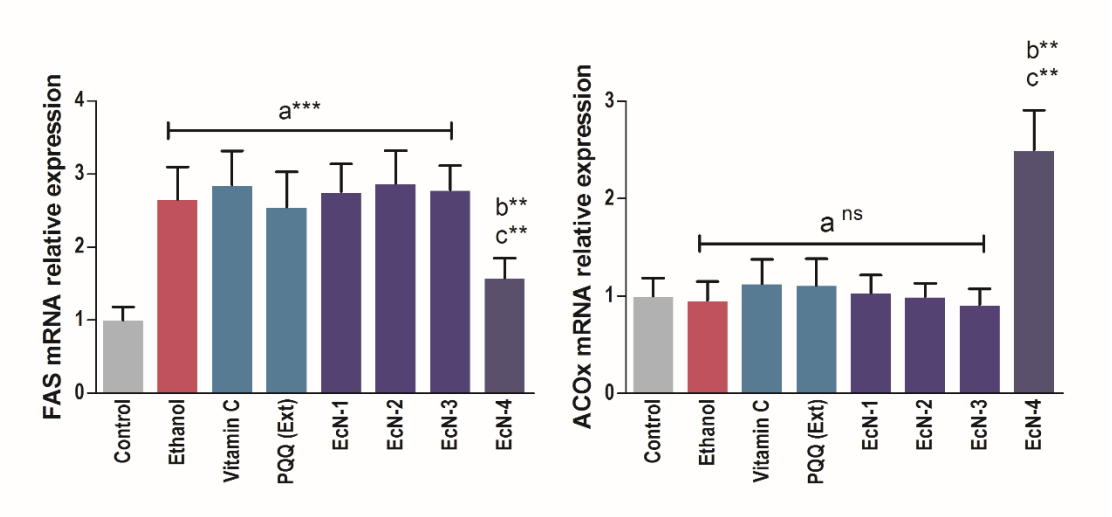

Figure 2.10 mRNA expression levels of Fatty acid synthase (A) and Acyl Coenzyme A oxidase (B) in hepatic tissue of rats co-treated with EcN-4 and chronic ethanol. Values are expressed as mean ± SD (n=6 each group). a***p≤ 0.001 compared to control group, b**p≤ 0.01 compared to ethanol group and c**p≤ 0.01 compared to PQQ (Ext) group.

Table 2.3 Body weight gain and lipid profile of chronic ethanol fed rats treated along with Vitamin C, PQQ and PQQ secreting EcN

Groups	Control	Ethanol	Vitamin C	PQQ (Ext)	EcN-1	EcN-2	EcN-3	EcN-4
Change in body weight (%)	60 ± 5.5	34 ± 8 a***	36 ± 7	34 ± 6	31 ± 7	33 ± 8	35 ± 8	51 ± 10 b* c*
Plasma (mg/dl)								
Total cholesterol	88.6 ± 5.2	130.2 ± 7.5 a***	127.6 ± 5.5	116.2 ± 4.2 b*	132.5 ± 4.3	128.1 ± 8.0	135.5 ± 4.5	84.8 ± 8.2 b** c*
Triglyceride	61 ± 4.1	125 ± 7.2 a***	123 ± 5.5	101.2 ± 9.0 b*	127 ± 6.5	124 ± 4.8	120 ± 8.4	64.3 ± 7.6 b** c**
HDL Cholesterol	21.2 ± 2.2	32.1 ± 3.1 a***	34.0 ± 3.0	28.0 ± 2.0 b*	32.5 ± 2.1	33.4 ± 3.4	32.7 ± 2.9	20.7 ± 3.2 b** c*
Liver (mg /g tissue)								
Total cholesterol	12.7 ± 0.29	18.2 ± 0.24 a***	17.9 ± 0.17	17.8 ± 0.22	18.1 ± 0.28	17.8 ± 0.27	18.2 ± 0.29	14.5 ± 0.22 b* c*
Triglyceride	11.8 ± 0.24	24.2 ± 0.80 a***	20.1 ± 0.50	21.7 ± 0.60	23.7 ± 0.78	24.8 ± 0.72	22.5 ± 0.65	14.6 ± 0.40 b** c**

Values are expressed in mean ± SEM (6 rats each group)

a***p≤0.001 compared to control group. b*p≤ 0.05 and b**p≤ 0.01 compared to ethanol group. c*p≤ 0.05 and c**p≤ 0.01 compared to PQQ (Ext) group.

2.3.6 PQQ concentration in feces and liver in rats treated with EcN-4 secreting PQQ

PQQ concentration estimated in fecal matter and in liver of EcN-4 co-treated rats, on the day of sacrifice, was found to be nearly 2 fold and 4 fold more than control and ethanol treated rats (**Table 2.4**). Rats co-treated with PQQ (Ext), Vitamin C, EcN-1, EcN-2 and EcN-3 had PQQ concentration similar to control and ethanol treated rats.

2.3.7 Colonic Short Chain Fatty Acid (SCFAs) concentration in chronic ethanol fed rats co-treated with EcN-4 secreting PQQ.

Rats co-treated with EcN-4 had significantly higher concentrations of colonic SCFAs as compared to control and those treated with ethanol. Butyrate, propionate and acetate were found to be increased by 124 %, 39 % and 7 %, respectively, in these rats when compared to only ethanol fed rats (**Table 2.5**). Other co-treatments did not alter colonic SCFAs concentration.

2.3.8 Effect of EcCFR16::*vgb-gfp* (*pqq*) secreting PQQ on antioxidant status and lipid profile in chronic ethanol treated rats.

EcCFR16 secreting PQQ had similar effect as EcN-4 secreting PQQ against chronic ethanol ingestion in rats **(Table 2.6)**.

Table 2.4 PQQ concentration in fecal matter and liver homogenate of chronic ethanol fed rats treated along with Vitamin C, PQQ and PQQ secreting EcN

Groups	Control	Ethanol	Vitamin C	PQQ (Ext)	EcN-1	EcN-2	EcN-3	EcN-4
Fecal n moles/ g fecal wet weight	0.756 ± 0.04	0.712 ± 0.06	0.790 ± 0.08	0.776 ± 0.02	0.70 ± 0.10	0.805 ± 0.07	0.763 ± 0.05	1.46 ± 0.05 a** b**
Liver picomoles/ g tissue	28.4 ± 5	26.6 ± 8	27.6 ± 6	36.2 ± 8	29.7 ± 5	24.8 ± 7	28.8 ± 4	112.6 ± 10 a** b**

Values are mean ± SEM (6 rats each group). a**p≤ 0.01 compared to control or ethanol group. b**p≤ 0.01 compared to PQQ (Ext) group.

Table 2.5 Short chain fatty acids (SCFAs) concentration in colonic matter of experimental rats.

Groups	Control	Ethanol	Vitamin C	PQQ (Ext)	EcN-1	EcN-2	EcN-3	EcN-4
Acetate	82.4 ± 2.4	90.1 ± 4.2 a*	89.2 ± 5.2	90.5 ± 4.4	88.8 ± 3.5	89.3 ± 3.7	92.1 ± 3.3	96.4 ± 4.1 b* c*
Propionate	19.1 ± 2.2	20.2 ± 1.9	18.8 ± 2.1	19.4 ± 1.8	21.2 ± 2.1	20.7 ± 1.8	19.6 ± 2.1	26.5 ± 2.4 b** c*
Butyrate	8.2 ± 0.5	8.0 ± 0.9	7.8 ± 1.0	8.1 ± 0.6	8.3 ± 0.8	7.5 ± 1.2	7.8 ± 1.3	18.4 ± 0.7 b** c**

Values are expressed as µ moles / g colonic content. Values are mean ± SEM (6 rats each group).
a*p≤ 0.05 compared to control group. b*p≤ 0.05 and b**p≤ 0.01 compared to ethanol group. c*p≤ 0.05 and c**p≤ 0.01 compared to PQQ (Ext) group.

Table 2.6 Effect of EcCFR16::*vgb-gfp* (*pqq*) secreting PQQ on antioxidant status and lipid profile in chronic ethanol treated rats.

Parameters	Control	Ethanol	EcCFR16-WT	EcCFR16:: *vgb-gfp*	EcCFR16::*vgb-gfp* (VC)	EcCFR16::*vgb-gfp* (*pqq*)
			Antioxidant parameters			
Hepatic LPO n moles MDA/ mg tissue	92.4 ± 31.8	380 ± 30.5 a***	386 ± 35.5	385 ± 28.5	390 ± 32.2	150 ± 35.5 b***
Hepatic GSH µ moles/ g tissue	11.95 ± 1.1	5.1 ± 1.2 a***	4.9 ± 1.1	5.2 ± 1.3	4.8 ± 1.3	10.6 ± 1.5 b***
Hepatic CAT U/ mg protein	183.5 ± 23.3	37.5 ± 14.5 a***	35.6 ± 12.8	38.6 ± 15.5	40.2 ± 18.6	165 ± 20.4 b***
Hepatic SOD U/ mg protein	7.1 ± 0.5	2.4 ± 0.3	2.3 ± 0.35	2.4 ± 0.5	2.3 ± 0.53	5.5 ± 0.6 b***
Blood LPO n moles MDA/ ml	46.08 ± 9.45	178.7 ± 9.8 a***	181.2 ± 7.5	177.4 ± 5.5	180.4 ± 8.3	68.5 ± 8.5 b***
RBC-CAT U/ mg protein	333.6 ± 46.5	110.4 ± 8.9 a***	115.3 ± 10.4	113 ± 8.5	112.8 ± 8.5	280.5 ± 12.5 b***
RBC-SOD U/ mg protein	0.74 ± 0.08	0.28 ± 0.09 a***	0.30 ± 0.07	0.31 ± 0.085	0.29 ± 0.095	0.67 ± 0.10 b***
Colon LPO n moles MDA/ mg tissue	52.1 ± 17.6	162 ± 5.4 a***	165 ± 6.7	158.8 ± 7.5	160 ± 8.2	58.5 ± 5.5 b***
Colon CAT U/ mg protein	25.7 ± 3.9	10.2 ± 2.2 a***	11.5 ± 3.2	10.5 ± 2.8	11.2 ± 2.8	21.7 ± 3.5 b***
SGPT U/ L	72.5 ± 22.2	170.5 ± 20.8 a***	175.4 ± 23.2	170 ± 21.2	169.5 ± 22.7	102.8 ± 18.6 b***
Total Bilirubin mg/dl	0.61 ± 0.05	1.14 ± 0.08 a***	1.13 ± 0.09	1.12 ± 0.10	1.2 ± 0.09	0.6 ± 0.09 b***

Body weight (BW) and lipid profile

% change in BW	60 ± 5.5	34 ± 8 a***	30 ± 8	35 ± 6	36 ± 9	55 ± 12 b*
Plasma TG	61 ± 4.1	125 ± 7.2 a***	125 ± 5.8	121 ± 5.2	123 ± 7.9	69.5 ± 9.8 b**
Plasma CHO	88.6 ± 5.2	130 ± 7.5 a***	138.5 ± 8.5	122.7 ± 8.5	128.5 ± 8.5	92.6 ± 9.4 b**
Plasma HDL	21.2 ± 2.2	32.1 ± 3.1 a***	30.5 ± 4.2	31.4 ± 4.5	34.4 ± 3.5	24.8 ± 4.3 b**
Hepatic TG	11.8 ± 0.24	24.2 ± 0.8 a***	25.5 ± 1	23.9 ± 0.9	26.2 ± 0.7	16.5 ± 0.7 b**
Hepatic CHO	12.7 ± 0.29	18.2 ± 0.2 a***	18.5 ± 0.42	18.2 ± 0.41	19.2 ± 0.33	16.5 ± 0.52 b*

Values are expressed in mean ± SEM (6 rats each group) a***p≤0.001 compared to control group. b*p≤ 0.05 and b**p≤ 0.01 compared to ethanol group.

2.4 Discussion

Ethanol toxicity results either directly from ethanol and its metabolite, acetaldehyde, or indirectly by metabolic consequences of ethanol oxidation such as increased ROS and decreased cytoplasmic NAD^+/NADH ratio. Ethanol induced free radical generation is mediated by cytochrome P450 II E1 (CYP2E1) and NADPH reductase dependent Microsomal Ethanol Oxidizing System (MEOS), and xanthine oxidase (Lieber, 1999). Acute ethanol ingestion increases CYP2E1 activity, inhibits glutathione peroxidase and glutathione reductase in the liver resulting in decreased GSH level and increased lipid peroxidation (Oh et al., 1997). Antioxidants play important role in preventing molecular and cellular damage caused by free radicals. Antioxidants such as Vitamin C and N-acetyl cysteine (NAC) have been shown to attenuate acute ethanol toxicity by lowering lipid peroxidation and GSH depletion up to a certain extent (Wang et al., 2006; Lee et al., 2013). PQQ has been shown to reduce acetaldehyde levels in blood after ethanol ingestion in rats (Hobara et al.1988). But its effect against ethanol toxicity has not been studied. PQQ has been shown to be most efficient antioxidant molecule and prevents cells from oxidative damage and injury in cell culture as well as rodent models (Rucker et al., 2009). *In vitro* cell free system suggests that PQQ scavenges singlet oxygen (Mukai et al., 2011), superoxide anions and hydroxyl radicals (Urakami et al., 1997). It also suppresses peroxynitrite formation by NO donors preventing neurotoxicity (Zhang and Rosenberg, 2002). The present study bespeaks that pre-treatment of vitamin C, PQQ and PQQ secreting EcN (EcN-4) prevents acute ethanol induced oxidative stress in rats. It is notable that PQQ treatment confers much more pronounced protective effect than vitamin C and EcN-4 treatment in against acute ethanol dose in rats. Since, many studies have shown the presence of PQQ in dietary components and mammalian tissues including liver (Kumazawa et al., 1992; Mitchell et al., 1999), we hypothesize that, PQQ transported to the liver after external PQQ treatment and secreted by EcN in the intestine, is responsible for attenuation of acute ethanol induced lipid peroxidation and GSH depletion in rats. EcN-4 pre-treatment (pre-colonization) is found to be less effective as compared to PQQ (Ext)

treatment, which indicates it is possible that the less PQQ concentration is achieved in the liver and the effect may be concentration dependent.

On the other hand, chronic ethanol ingestion not only increases ROS but also diminishes antioxidant enzyme activities resulting in oxidative cellular damage and morphological changes in all the major tissues (Koch et al., 2004; Gao and Bataller, 2011). In normal metabolism, ROS generation is controlled by antioxidant defense mechanisms. In contrast to acute studies, studies demonstrating ameliorative effects of antioxidants and other bioactive compounds against chronic ethanol induced oxidative damage have been limited. The present study of 10 weeks, weekly treatment of PQQ secreting probiotic *E. coli* Nissle 1917 (EcN-4) completely attenuates chronic ethanol induced oxidative damage in hepatic, blood, renal and colonic tissues of rats. In contrast to acute ethanol exposure, EcN-4 treatment is more effective than externally given PQQ in chronic ethanol exposure. Significant increase in PQQ concentration in the liver and fecal matter of rats treated with EcN-4 signifies that PQQ secreted by this probiotic endogenously in the gut is transported to the major organs and acts against free radicals generated after ethanol metabolism. It is very evident from the study that weekly PQQ (Ext) treatment (10 mg/kg body weight) does not have ameliorative effect against chronic ethanol toxicity, except a marginal amelioration in colonic and renal lipid peroxidation. Moreover, a marginal but significant reduction in lipid peroxidation was observed in rats treated with EcN, EcN-2 and EcN-3. This could be a consequence of EcN preventive function. EcN is known to prevent damage of the tight junctions and maintains intestinal integrity through upregulation and re-localization of tight junction proteins (zonula occludens-1 and zonula occludens-2), and inhibition of protein kinase C isoform (Zyrek et al., 2007). The preventive effect of *E. coli* Nissle 1917 against chronic ethanol toxicity seems to be localized to colonic tissues only. It is important to note that vitamin C weekly treatment (350 mg/kg body weight) was ineffective against chronic ethanol induced oxidative damage.

Chronic alcohol ingestion is also associated with alcoholic fatty liver and hyperlipidemia (Baroana and Lieber, 1979; Gao and Bataller, 2011). Increase

in lipid content of liver and blood are the result of altered lipid metabolism induced by chronic ethanol intake. Ethanol induces fatty acid biosynthesis by activation of sterol responsive element binding protein 1 (SREBP-1) and up regulation of FAS gene expression (You et al., 2002). Chronic ethanol intake also causes malnutrition, because of which weight loss is often seen at later stages of ethanol treatment. Apart from antioxidant activity, PQQ is known to interact with intracellular signal transduction pathways in mammalian cells (Rucker et al., 2009; Feng et al 2013). Bauerly et al. (2011) showed that PQQ interacts with signaling pathways involved in lipid and mitochondrial metabolism. PQQ deprived diet leads to hyperlipidemia and up regulation of lipid biosynthesis enzymes in mice. When PQQ in the diet is restored, lipid profile and expression levels of genes also get restored to normal levels. Moreover, they showed that administration of PQQ results in more than 2 fold decrease in triglyceride levels in rat model of type-2 diabetes. In the current study, PQQ secreting EcN (EcN-4) treatment significantly reduced blood and liver lipid levels and improved chronic ethanol induced weight loss with concomitant reduction of FAS gene expression and elevation of ACOx gene expression. The results are in agreement with previous findings and reports attributing PQQ functions in mammals (Rucker et al., 2009; Bauerly et al., 2011).

EcN-4 treatment also resulted in increase in colonic SCFAs (Butyrate, propionate and acetate). PQQ acts as cofactor for the oxidation of glucose to gluconic acid in many gram negative bacteria, especially *E. coli* (Rucker et al., 2009), and gluconic acid is known to increase colonic SCFAs (mainly butyrate) in rats (Kameue et al., 2004). Thus, we hypothesize that increase in colonic SCFAs is mediated by gluconic acid produced by EcN-4 as well as native intestinal *E. coli* strains in rats treated with PQQ secreting EcN (EcN-4). SCFAs are also involved in regulation of lipid metabolism. Acetate suppresses body fat through up-regulation of lipid oxidizing enzymes in liver (Kondo et al., 2009), propionate lowers fatty acid content in liver and plasma (Al-Lahham et al., 2010) and butyrate regulates cholesterol synthesis, stimulates β- oxidation of fatty acids and peroxisome proliferation (Canani et al., 2011). Thus, amelioration of ethanol induced hyperlipidemia and modulation of hepatic

gene expression in EcN-4 treated rats is the result of the combined effects of both PQQ and SCFAs.

Vitreoscilla hemoglobin (VHb) protein,encoded by *vgb* gene, has been shown to improve bacterial growth under microaerophilic condition and also possesses peroxidase activity (Kallio *et al.,* 1994; Kvist et al., 2007). However, EcN genomic integrant of *vgb* and *gfp* genes (EcN-2) here did not show any additional benefit as compared to EcN.

In conclusion, probiotic *E. coli* Nissle 1917 can be used for efficient and constant delivery of PQQ as only native and genetically modified Gram negative bacteria are known to produce PQQ. High amount of PQQ in the intestine and other body tissues prevents complications caused by chronic ethanol ingestion in rats and could also be effective in preventing alcoholic liver disease. Pandey et al. (2014) recently demonstrated ameliorative effect of PQQ secreting *E. coli* CFR16 against 1,2-dimethyl hydrazine induced toxicity in rats. EcN has been shown to be effective against stress and inflammation in inflammatory bowel disease. Genetically modified *E. coli* Nissle 1917 have been constructed for secretion of azurin (anti-carcinogen) and defensins (host defence peptide) (Seo et al., 2012; Zhang et al., 2012). Thus the present study demonstrates that constant endogenous secretion and transport of PQQ in mammals can be achieved by genetic manipulation of *E. coli*, which will minimize, if not eliminate, the dietary PQQ requirement. Benefits of PQQ are more pronounced by the use of PQQ secreting EcN. Since EcN strains (lacking PQQ secreting capability) seems to be inefficient against chronic ethanol exposure, the effectiveness of EcN-4 could be attributed to PQQ. Hence, weekly dose of PQQ secreting EcN and daily dose of PQQ could have similar ameliorative effect against chronic ethanol toxicity.