

# Chapter 3

**Evaluating the effect of probiotic *E. coli* strains producing pyrroloquinoline quinone (PQQ) on fructose induced metabolic effect and antioxidant status in Charles Foster rats.**

### 3. Introduction

Metabolic syndrome and its associated pathologies are characterized with increased levels of reactive oxygen species (ROS) (**Hutcheson et al. 2012**). In healthy state, levels of these ROS molecules are maintained within optimum range by enzymatic (superoxide dismutase, glutathione, catalase, peroxidase) and non-enzymatic (vitamins C and E) antioxidants. However, increased oxidant capacity with concomitant decreased antioxidant status is attributed to an unbalanced environment leading to oxidative stress in diseased state. Presence of high levels of ROS contributes to increased oxidation of carbohydrates, lipids, proteins and causes toxic effects on cells and tissues. In addition, ROS is implicated in the development and progression of cardiovascular disease as well as a major mechanism behind micro- and macro-vascular complications in the metabolic syndrome.

Despite of the efficient endogenous antioxidant system, humans depend on variety of antioxidant molecules present in the diet to maintain free radical concentrations at low levels (**Pietta et al. 2006**). Synthetic antioxidants are frequently used in food and pharmaceutical products (**Sökmen et al. 2004**). However, their use raises consumer questions regarding side effects and potential toxicities. In recent years, probiotics have gained significant consideration as natural therapeutic agents for alleviating risk oxidative stress and metabolic complications associated with metabolic syndrome (**Kelishadi et al 2013**). However, effectiveness of most probiotics is limited by variable colonization and survival ability in gastrointestinal tract (**Verna et al. 2010**).

PQQ is one of the most potent water soluble, antioxidant molecule with remarkable ability to undergo redox cycling of ~20,000 times (**Rucker et al. 2009**). It is highly stable at variable temperatures and pH. Plant and animal cells, biological fluids such as milk and many foods contain PQQ in pM to nM range. Apart from antioxidant property of PQQ, it possesses cardio- and neuro-protectant properties. Dietary PQQ deprivation in animal models has shown immune dysfunction, abnormal development and decreased reproductive performance. Approximately, 2–4 percent of the total genes respond to changes in PQQ status depending on dietary conditions or

pharmacologic administration. Gene array analysis showed that PQQ alters the expression of wide range of genes, most notably the genes involved in mitochondrial-related functions which revert to normal levels upon PQQ repletion (**Misra et al. 2012**). Mice and rats receiving PQQ deficient diet have shown 20–30% reduction in the relative amount of mitochondria in liver, lower respiratory control ratios and lower respiratory quotients (RQ) than mice receiving PQQ-supplemented diet. In addition, PQQ is also known to modulate signal transduction pathways involved in cellular stress, mitochondriogenesis, cell signaling and MAP kinase pathways, and transport of metabolites (**Rucker et al. 2009**).

Essential dietary requirement of PQQ in mice suggests that natural gut microbiota do not possess biosynthesis genes to synthesize PQQ (**Rucker et al. 2009**). PQQ is a cofactor for many bacterial dehydrogenases which are predominant in Gram negative bacteria. However, *E. coli* genome does not encode for PQQ biosynthesis genes (**Rucker et al. 2009**). Incorporation of PQQ biosynthesis genes in *E. coli* resulted in secretion of PQQ in high amounts (**Yang et al 2010**). EcN is a well characterized probiotic, possesses fitness factors *i.e.* siderophores, microcins and synthesis of peptide/polyketide hybrids (**Sonnenborn et al. 2009**). EcN genome also does not have PQQ biosynthesis genes (**Cress et al. 2013**).

*Vitreoscilla* haemoglobin (VHb) is known to increase cell survival and cell growth. Besides, it can also affect expression of hundreds of genes in *E. coli* associated with energy metabolism, central intermediary metabolism and cellular processes as evident from proteomic and microarray analyses of recombinant *E. coli* expressing *vgb* gene (**Stark et al. 2012**). Several studies have demonstrated that VHb can alter antioxidant enzymes status and it also help to cope up with oxidative stress.

High amount of fructose in diet results in metabolic disorders chiefly because of the uptake mechanisms, lack of feedback regulation in fructose metabolic pathway and oxidative stress (**Miller et al. 2008**). Fructose is consumed as bulk sweetener in western diets. Moreover, increased uptake of total fructose and high fructose corn syrup has increased risk factors associated with obesity and diabetes in past two decades. Therefore, short and long term effects of fructose consumption have raised

serious concern in humans because of several factors: First, sucrose was replaced to large extent by high fructose corn syrup (HFCS) in carbonated beverages during 1980s, particularly in North America. Epidemics of obesity have risen in parallel with intake of soft drink containing HFCS (**Bray et al. 2004**). Secondly, increased uric acid levels, plasma triglycerides (TG) and VLDL-TG; LDL particle size in overweight school children was correlated with high fructose intake (**Nakagawa et al. 2006; Aeberli et al. 2007; Bray et al. 2007**). Additionally, fructose forms intermediate Schiff base with the  $\epsilon$ -amino group of lysine residue of proteins. Schiff base is further hydrogenated to form an irreversible Heyns product resulting in the generation of superoxide radical ( $O_2^{\bullet-}$ ) and inactivates protein by fructosylation (**Nagai et al. 2002; Bose et al. 2008**). For assessing therapeutic of potential against fructose induced metabolic syndrome, rats fed with high fructose diet are established models (**deMoura et al. 2009**).

The aim of the present study was to assess the efficacy of probiotic EcN:: *vgb-gfp* (*pqq*) on endogenous delivery of PQQ to liver and its protective effect on hyperlipidemia, hyperglycemia, oxidative stress and colonic short chain free fatty acid in fructose fed rats.

## 3.1 Material and Methods

### 3.1.1 Animals, diet and experimental design

Adult albino male Charles Foster rats (180-200 g) were used for animal studies. All the rats were maintained in controlled condition as per committee guidelines *i.e.* temperature ( $25 \pm 1$  °C), relative humidity (45.5 %) and photoperiod cycle (12 h light: 12 h dark). Free access to food and water was provided as per recommended by committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines of animal ethical committee of M. S. University of Baroda, India, Registration number 938/A/06/CPCSEA. Diet composition is according to **Singh et al. (2014)**.

For present study rats were divided into six different groups ( $n=6$ ) as follows: Group I received pellet diet and served as control group, Group II received pellet diet and 20 % fructose in drinking, Group III received pellet diet, 20 % fructose in drinking water and EcN, Group IV received pellet diet, 20 % fructose in drinking water and EcN-2, Group V received pellet diet, 20 % fructose in drinking water and EcN-3, Group VI received pellet diet, 20 % fructose in drinking water and EcN-4. Probiotics ( $10^9$  colony forming unit (CFU)) was supplemented to all groups except Group 1 and 2 once per week till six months.

### 3.1.2 Plasmid, Bacterial strains and culture condition

EcN (Mutaflor®) was obtained from Dr. Rer. Nat. Ulrich Sonnenborn as a generous gift and it is commercially available from Ardeypharm GmbH, Loerfeldstrabe 20, Herdecke, Germany. All the plasmid constructs and bacterial strains used in the present study are summarized in **Table 3.1**. *pqq* gene cluster of *Pseudomonas fluorescens* B16 was used in the study (GenBank Accession No. *pqqA*: CAA60731; *pqqB*: YP\_350886; *pqqC*: YP\_350887; *pqqD*: YP\_350888; *pqqE*: YP\_350889; *pqqF*: YP\_350884; *pqqH*: YP\_350893; *pqqI*: YP\_350892; *pqqJ*: YP\_218683; *pqqK*: NP\_436416; *pqqM*: YP\_350890 (**Choi et al., 2008**). Bacterial strains used in the study were cultured in Luria Broth (LB) overnight at 37 °C. For probiotics supplementation to different rat groups, overnight grown culture of different strains was re-inoculated in fresh L.B tubes to achieve final CFU of  $10^9$  per

ml. One ml of fresh culture (CFU of  $10^9$ /ml) was taken from the tube, centrifuged and washed twice with normal saline before oral administration to rats.

### 3.1.3 PQQ extraction and quantification

PQQ extraction and quantification from fecal and liver tissue was carried out as described by **Pandey et al. (2014)**.

### 3.1.4 Preparation of cell lysate and tissue homogenates

Blood was collected by orbital sinus puncture in EDTA coated and normal tubes followed by centrifugation at 1500 g for 10 min. Plasma and serum was collected separately in fresh tubes and stored in  $-80\text{ }^{\circ}\text{C}$  till use. Pack cell volume (PCV) was washed thrice with normal saline prior to lysis in ice cold water. Cell lysate so obtained was centrifuged at 15,000 g for 10 min and fresh supernatant was used for enzyme assays. Liver was collected and washed with PBS immediately after sacrificing rats. Liver homogenates were prepared in different buffers for antioxidant enzyme activity.

### 3.1.5 Biochemical assays

Superoxide dismutase (SOD) activity was measured by method which is based on auto-oxidation of pyrogallol in presence of oxygen (**Marklund et al. 1974**). Presence of SOD inhibits autoxidation of pyrogallol and it can be monitored spectrophotometrically at 420 nm. SOD activity of samples was reported as units/mg protein. Catalase (CAT) activity was monitored by measuring rate of disappearance of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) spectrophotometer at 240 nm (**Beers et al. 1952**). Unit enzyme activity is defined as 1  $\mu\text{mole}$  of hydrogen peroxide consumed per minute, and the specific activity is reported as units/mg protein. Function readout of reduced glutathione (GSH) was performed as described by **Beutler et al. (1969)**. Lipid peroxidation (LPO) was estimated by measuring levels of MDA at 412 nm as described by **Buege and Aust (1978)**.

Serum lipid profile (triglyceride, HDL-cholesterol and total cholesterol) and uric acid were measured as per manufacturer protocol of Beacon Diagnostics Pvt. Ltd., Navsari, India). Hepatic triglyceride and free cholesterol levels were measured as

described by Carr et al. (1993)<sup>26</sup>. Oil red O staining was carried out by the method of Lillie et al. (1943).

### 3.1.6 Colonic short chain fatty acid extraction and estimation

Immediately after sacrificing rats, colonic content was collected in sterile tube. Approximately 0.2 g of samples was diluted in water to achieve ratio of 1:8 (w/v). Diluted samples were vortexed; homogenized and centrifuged for 10 min at 10,000g. Clear supernatant was filtered through 0.2 µm cellulose acetate membrane and stored in deep freezer until HPLC was performed. SCFAs concentrations were quantified by using HPLC. Briefly, 50 µl of extracted fecal samples were injected directly into HPLC System (Shimadzu LC-10AD Liquid Chromatography) with Shimadzu SPD-6A UV-VIS detector (Shimadzu, Kyoto, Japan). Separation of SCFA was carried out by ionic exchange resin, Aminex HPX-87H column, (300 x 7.8 mm, Bio-Rad Laboratories, Richmond, USA) at 65 °C. Compounds were detected using UV detector set at 210 nm and 0.01 N H<sub>2</sub>SO<sub>4</sub> was used as a mobile phase at a flow rate of 0.6 ml/ min.

### 3.1.7 FAC and AOC mRNA expression and qRT-PCR in hepatic tissue

RNA extraction from tissue was carried out with Trizol reagent (Invitrogen) followed by generation of cDNAs from 1 µg total RNA as per manufacturers protocol (Reverse Transcription Kit, Applied Biosystems, Foster City, CA). Following primers were used for Fatty acid synthase and Acyl Co-enzyme A gene  
ACCTCATCACTAGAAGCCACCAG (forward) and  
TGGTACTTGGCCTTGGGTTTA (Reverse), and  
CCCAAGACCCAAGAGTTCATTC (Forward) and  
TCACGGATAGGGACAACAAAGG (Reverse), respectively. PCR coupled with SYBER green (Applied biosystems, Foster city, California, U.S.) was performed using ABI Quant Studio TM 12K flex Real Time PCR system Each sample analysis was performed in duplicates by using software provided by with the thermocycler (Quant Studio TM, Foster city, California, U.S.A.). Ct represents the mean cycle time of the linear part in the curve.

### 3.1.8 Statistical analysis

All values are expressed as means  $\pm$  SD. Differences in lipid peroxidation and antioxidant enzymes (SOD, CAT and GSH) among six different groups were evaluated using the one-way ANOVA followed by Bonferroni comparisons. Differences were considered significant at  $P < 0.05$ . Calculations were performed using commercially available statistical software packages (Graph Pad PRISM Version 5.0 La Jolla, CA 92037, USA).

## 3.2 Results

### 3.2.1 Genomic integration of *gfp* and *vgb* in EcN

Recombinant pGRG36 plasmid harbouring *gfp-vgb* genes were used to transform wild type EcN and selection of transformants were carried out on ampicillin containing plates. Genomic integration of *gfp* and *vgb* in EcN was carried out by Tn7 mediated genomic integration. EcN genomic integrants were selected by loss of ampicillin resistance and confirmed by gene specific PCR (Fig. 3.1). Growth profile of WT EcN and EcN-2 did not display any significant difference. In addition, co-inoculation of WT EcN and EcN-2 not exhibited any significant differences in GFP positive and GFP negative colonies (Fig 3.2).

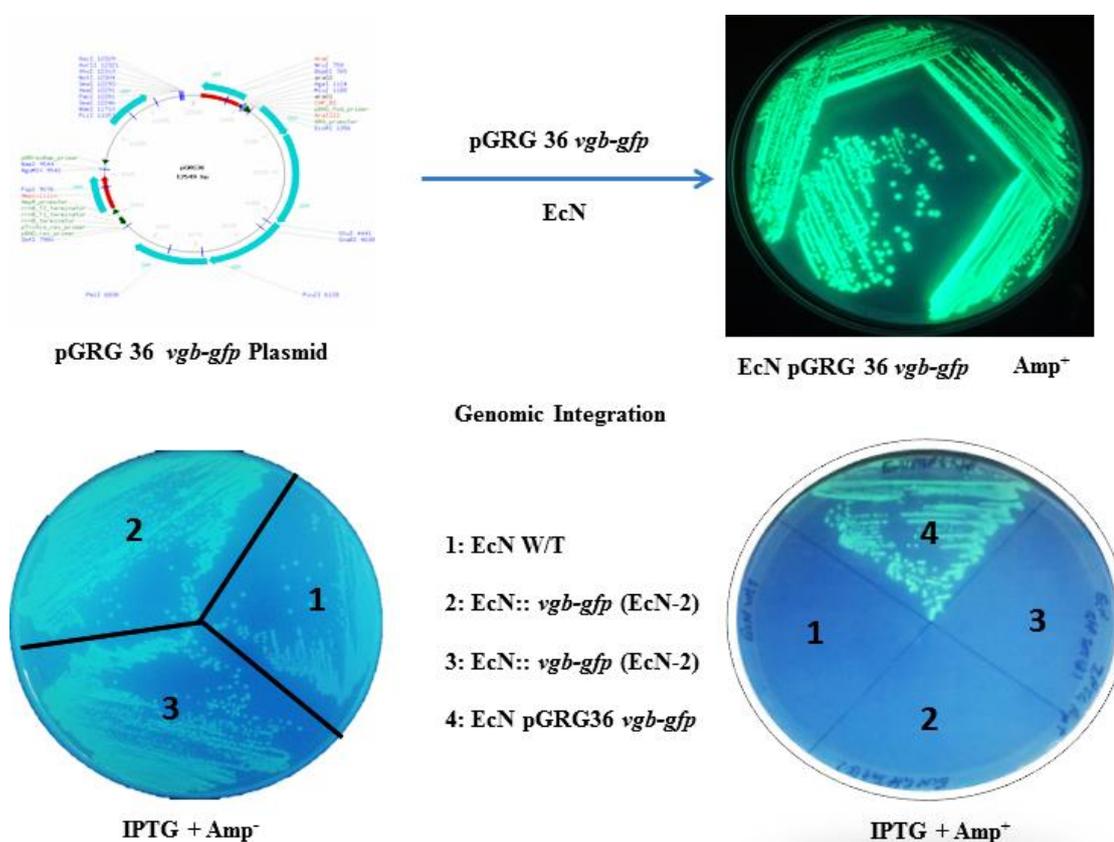
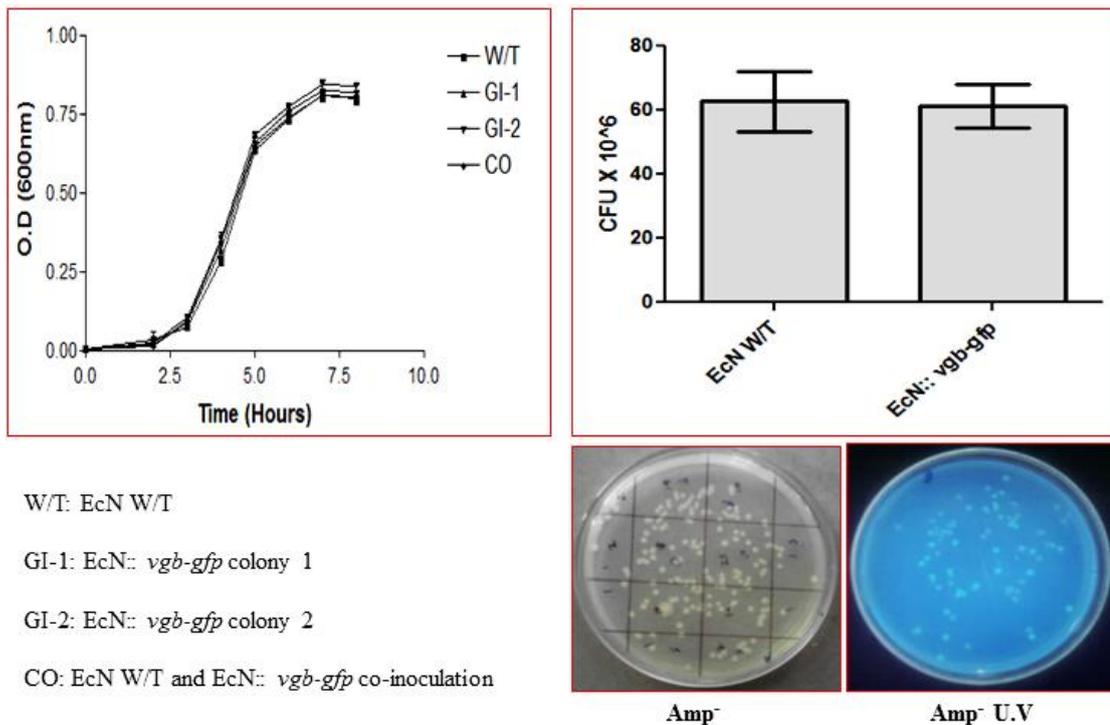


Fig. 3.1: Genomic integration of *gfp* and *vgb* in EcN.



**Fig. 3.2: Growth curve of EcN W/T and EcN:: *vgb-gfp* alone and in co-inoculation.**

### 3.2.2 Colonization of EcN (*gfp*) and EcN:: *vgb-gfp* in rat intestine

Colonization potential of modified EcN-2 was evaluated by flow cytometry after feeding single dose of  $10^9$  CFU each of EcN (*gfp*) and EcN-2 to rats. EcN-2 exhibited improved colonization potential in comparison to EcN (*gfp*) (**Fig 3.3**).

### 3.2.3 Confirmation and characterization of EcN:: *vgb-gfp* (*pqq*)

Recombinant pBBR1MCS-II plasmid harbouring *pqq* gene cluster was used to transform EcN-2. Confirmation of PQQ secreting EcN-2 was carried out by restriction digestion and PQQ quantification. Functionality of PQQ gene cluster was monitored on Tris Buffered media, EcN-4 exhibited presence of red colour which was absent in EcN W/T. PQQ quantification in M9 minimal media was found to be  $\sim 1.7 \mu\text{g}$  PQQ/ml culture (**Fig. 3.4**).

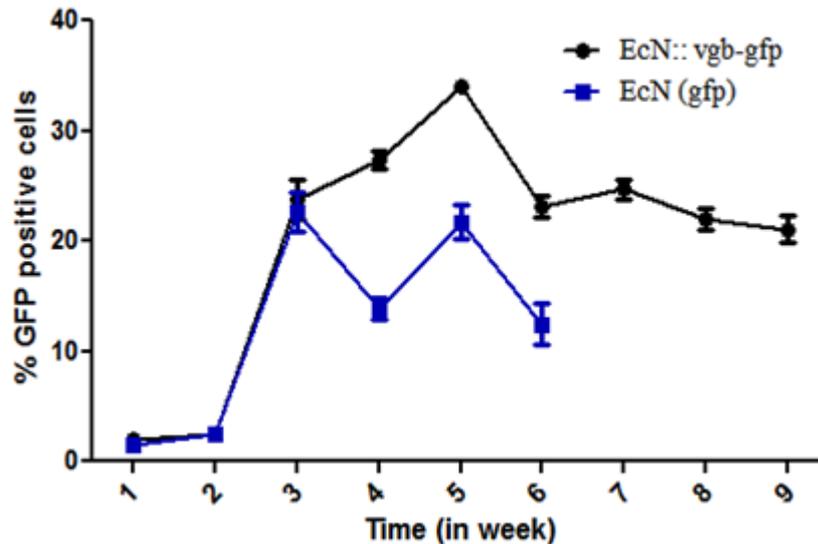
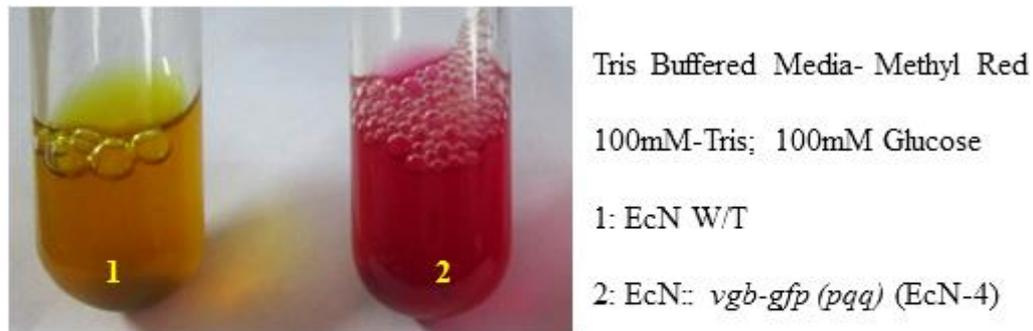


Fig. 3.3: Colonization of EcN (*gfp*) and EcN:: *vgb-gfp* in rat intestine.



EcN:: *vgb-gfp* (*pqq*) (EcN-4):

PQQ quantification in M9 minimal media: ~1.7  $\mu$ g PQQ/ml culture

Fig. 3.4: Functionality of *pqq* gene cluster in Tris buffered media and PQQ quantification.

### 3.2.4 Food intake, body weight gain, fasting glucose levels, serum insulin and uric acid levels

Food intake of all rat groups receiving pellet diet and 20 % fructose in drinking water was found to be similar except in group 1 rats which were receiving only pellet diet and showed significantly high chow consumption (**Table 3.2**). Moreover, chronic consumption of 20 % fructose in drinking water significantly increased body weight of group 2 rats by 56 % in comparison with that of the Group 1 rats. Albeit body weight of rats receiving EcN, EcN-2 and EcN-3 was similar to the Group 2 rats while rats receiving EcN-4 showed ~28 % reduction in body weight. Fasting serum glucose

levels of Group 2 rats was found to be 60 % higher in comparison with Group 1 rats. In contrast, Group 6 rats supplemented with EcN-4 showed ~ 17 % decrease in serum glucose levels. As observed earlier in the case of body weight gain, rats administered with EcN, EcN-2 and EcN-3 did not exhibit any significant change with respect to serum glucose levels. Moreover, serum insulin levels were likewise found to be lowered in rats supplemented with EcN-4. In addition, serum uric acid levels were ~ 2 fold higher in Group 2, 3, 4 and 5 in comparison to the control rats. Administration of EcN-4 maintained the serum uric acid levels within normal range. To check the functionality of PQQ gene cluster *in-vivo* we estimated PQQ concentration in Group 6 rats, which were receiving EcN-4. Marked increase in PQQ concentration by ~ 2 fold and ~ 3 fold was observed in the rat's fecal matter and liver, respectively (**Table 3.3**). Similar results were also found in case of Ec16.

### 3.2.5 Serum and hepatic lipid profile

An elevated serum triglyceride level, a characteristic of metabolic syndrome, was observed with ~76% increase in Group 2 rats, along with a marked ~ 72% decrease in serum HDL levels (**Fig. 3.5 and 3.6**). Similarly, elevated levels of serum triglyceride and reduced serum HDL were found in all other groups except Group 6 rats which showed reduced triglyceride levels to almost ~ 35 % with concomitant increase in HDL levels by more than 2 fold than Group 2 rats. However, serum cholesterol levels did not show any significant change in all the rat groups. To further evaluate whether elevated serum triglyceride levels correlates with hepatic triglyceride levels, hepatic lipid profile was monitored. Hepatic triglyceride levels were found to be ~ 34 % higher in Group 2 rats which correlate with increased serum triglyceride levels (**Table 3.4**). Administration of EcN-4 significantly reduced (~ 20 %) hepatic triglyceride levels in Group 6 rats. In contrast, no significant difference was observed in rat groups receiving EcN, EcN-2 and EcN-3 dose, respectively. Cholesterol levels also showed up to 39% decrease in Group 6 rats, which was not the case with Group 3, 4 and 5 rats. To reconfirm these results we performed Oil red-O-staining for hepatic tissues of Group 1, 2 and 6 rats. High degree of lipid droplet accumulation was found in Group 2 rats while Group 6 rats receiving EcN-4 supplementation showed a significant reduction in lipid droplet accumulation which

was correlated with decreased serum and hepatic triglyceride levels (**Fig. 3.7**). Similar results were also found in case of Ec16.

### 3.2.6 Serum and hepatic LPO and antioxidant enzyme activities

LPO is a marker for oxidative stress and it was found to be ~ 2-3 folds higher in the blood of rats fed with 20 % fructose without probiotic supplementation in comparison with the control rat group receiving only pellet diet (**Fig. 3.8 and 3.9**). EcN, EcN-2, and EcN-3 administered rats did not show any significant decrease in blood LPO levels. However, normal levels of LPO were found in rats receiving EcN-4. Antioxidant enzymes, SOD and CAT, showed ~2 fold reduced activity in Group 2 rats when compared with Group 1 rats which were receiving only pellet diet (**Fig. 3.8 and 3.9**). Similar decrease in these antioxidant enzyme activities was found in Group 3, 4 and 5. In contrast, supplementation with EcN-4 restored SOD and CAT enzyme activities in normal range in Group 6 rats. Blood GSH levels also showed similar trend as observed in the case of SOD and CAT.

As fructose is metabolized solely in liver we further investigated LPO and antioxidant status in hepatic tissue. LPO levels in hepatic tissue of Group 2 rats were increased by ~2-3 folds in comparison to the Group 1 rats. As observed in the earlier cases, administration of EcN-4 significantly restored LPO to its normal levels. However, EcN, EcN-2 and EcN-3 supplementation did not exhibit any substantial effect in the rats. Hepatic SOD and CAT enzyme activities also showed a ~2 fold decrease which was restored to normal range in the rats administered with EcN-4. A similar increase in the hepatic GSH level was found in these rats of Group 6. Similar results were also found in case of Ec16.

### 3.2.7 Colonic short chain fatty acids (SCFA)

PQQ is a bacterial co-factor for PQQ dependent glucose dehydrogenase. EcN-4 secreting PQQ can hence significantly produce gluconic acid which can further be metabolized to SCFA by the gut microflora. Colonic acetate, propionate and butyrate levels were found to be decreased in Group 2 rats in comparison with Group 1 rats. Supplementation of EcN-4 resulted in a substantial increase in all three SCFA *i.e.* acetate propionate and butyrate (**Table 3.5**).

### 3.2.8 mRNA expression of FAS and ACOx in hepatic tissue

Finally, we monitored mRNA expression of FAS and ACOx in hepatic tissue as they play a crucial role in fatty acid metabolism. An increased expression of FAS and a reduced ACOx expression were observed in Group 2 rats (**Fig. 3.10**). On the other hand, Group 6 rats, which were supplemented with PQQ producing EcN demonstrated a reduced level of FAS expression and an increased ACOx expression. However, no significant changes in this instance were observed in Group 3, 4 and 5 rats.

Plasmid / strains	Characteristics	Reference
Plasmids		
pBBR1-MCS2	pBBR1-MCS2 Broad-host-range plasmid, <i>LacI</i> , multiple cloning site in <i>LacZ</i> ; <i>Kan<sup>r</sup></i>	Kovach et al. 1995
pOK51	13.3-kb <i>Bam</i> HI fragment from pOK40 cloned into pBluescriptII SK <sup>+</sup>	Choi et al. 2008
pBBR1-MCS2 <i>pqq</i>	pBBR1-MCS2 containing <i>Pseudomonas fluorescens</i> B16 <i>pqq</i> gene cluster at <i>Bam</i> HI site	Pandey et al. 2014
Bacteria		
EcN	<i>Escherichia coli</i> Nissle 1917 (EcN) (Probiotic strain)	Singh et al. 2014
EcN-2	EcN strain with genomic integration of <i>vgb</i> and <i>gfp</i> genes	Singh et al. 2014
EcN-3	EcN-2 harbouring pBBR1-MCS2 plasmid	Singh et al. 2014
EcN-4	EcN-2 harbouring <i>vgb-gfp</i> with pBBR1-MCS2 <i>pqq</i> plasmid	Singh et al. 2014
<i>E. coli</i> CFR 16 (Ec 16)	<i>E. coli</i> CFR 16 (Probiotic rat intestinal isolate)	Kumar et al., 2009
<i>E. coli</i> CFR 16:: <i>vgb-gfp</i> (Ec16-2)	<i>E. coli</i> CFR 16 strain with genomic integration of <i>vgb</i> and <i>gfp</i> genes	Kumar thesis, 2012
<i>E. coli</i> CFR 16:: <i>vgb-gfp</i> Vector (Ec16-3)	<i>E. coli</i> CFR 16 strain with genomic integration of <i>vgb</i> and <i>gfp</i> genes harbouring pBBR1-MCS2 plasmid	Pandey et al., 2014
<i>E. coli</i> CFR 16:: <i>vgb-gfp</i> ( <i>pqq</i> ) (Ec16-4)	<i>E. coli</i> CFR 16 strain with genomic integration of <i>vgb</i> and <i>gfp</i> genes harbouring pBBR1-MCS2 <i>pqq</i> plasmid	Pandey et al., 2014

**Table 3.1: Bacterial strains and plasmids.**

Groups	Body weight gain (gm)	Food intake (gm/day)	Fasting glucose (mg/dl)	Serum insulin ( $\mu$ L)	Serum uric acid (mg/dl)
Control	52.8 $\pm$ 4.86	12.25 $\pm$ 1.69	95.60 $\pm$ 6.46	1.23 $\pm$ 0.21	0.89 $\pm$ 0.06
Fructose control	82.6 $\pm$ 14.02**	8.54 $\pm$ 2.23*	153.81 $\pm$ 7.61***	3.12 $\pm$ 0.25**	1.64 $\pm$ 0.19***
EcN	77 $\pm$ 7.58**	8.96 $\pm$ 2.14*	148.55 $\pm$ 10.74***	2.92 $\pm$ 0.35**	1.53 $\pm$ 0.097***
EcN-2	72.8 $\pm$ 10.82**	8.77 $\pm$ 1.65*	144.20 $\pm$ 9.46***	3.01 $\pm$ 0.15**	1.50 $\pm$ 0.13***
EcN-3	74.2 $\pm$ 8.75**	8.74 $\pm$ 1.35*	151.32 $\pm$ 10.64***	2.98 $\pm$ 0.21**	1.48 $\pm$ 0.16***
EcN-4	59 $\pm$ 7.31##	9.32 $\pm$ 2.25*	127.29 $\pm$ 4.81**##	1.43 $\pm$ 0.35##	0.93 $\pm$ 0.021###
Groups	Body weight gain (gm)	Food intake (gm/day)	Fasting glucose (mg/dl)	Serum insulin ( $\mu$ L)	Serum uric acid (mg/dl)
Control	53.7 $\pm$ 4.71	11.31 $\pm$ 1.77	95.60 $\pm$ 6.46	1.18 $\pm$ 0.32	0.89 $\pm$ 0.10
Fructose control	80.7 $\pm$ 15.05**	8.90 $\pm$ 2.56*	157.23 $\pm$ 8.21***	2.97 $\pm$ 0.27**	1.67 $\pm$ 0.21***
Ec16	78.24 $\pm$ 6.91**	8.98 $\pm$ 2.57*	151.53 $\pm$ 13.13***	3 $\pm$ 0.32**	1.55 $\pm$ 0.12***
Ec16-2	73.9 $\pm$ 11.71**	8.88 $\pm$ 2.87*	153.54 $\pm$ 11.45***	3.09 $\pm$ 0.17**	1.59 $\pm$ 0.17***
Ec16-3	75.6 $\pm$ 9.13**	9.37 $\pm$ 2.23*	149.12 $\pm$ 12.45***	3.12 $\pm$ 0.21**	1.61 $\pm$ 0.21***
Ec16-4	58.34 $\pm$ 8.51##	9.21 $\pm$ 1.98*	125.56 $\pm$ 5.64**##	1.49 $\pm$ 0.35##	0.96 $\pm$ 0.29###

**Table 3.2: Effect of modified EcN and Ec16 on body weight gain, food intake, fasting glucose, serum insulin and serum uric levels of rats. Values are expressed as mean  $\pm$  SEM (n = 6 each group). \*P  $\leq$  0.05, \*\* P  $\leq$  0.01 and \*\*\* P  $\leq$  0.001 compared with control group. ## P  $\leq$  0.01 compared with fructose control group.**

Groups	Control	Fructose control	EcN	EcN-2	EcN-3	EcN-4
Fecal (nM /g fecal wet weight)	0.769±0.10	0.756±0.09	0.701±0.11	0.781±0.13	0.733±0.04	1.51±0.09**
Liver (pmole/g tissue)	26.29±5.23	28.21±3.39	32.99±6.67	24.26±2.95	27.48±5.05	115.87±8.89***

Groups	Control	Fructose control	Ec16	Ec16-2	Ec16-3	Ec16-4
Fecal (nM /g fecal wet weight)	0.754±0.13	0.761±0.16	0.745±0.21	0.755±0.19	0.759±0.14	1.61±0.23**
Liver (pmole/g tissue)	29.23±6.6	31.56±7.21	28.78±5.94	32.34±8.32	27.75±6.01	121.56±10.21***

Table 3.3: PQQ concentration in liver tissue and fecal sample of rats fed with modified strains of EcN and Ec16 at the end of experiment: Values are expressed as mean ± SEM (n = 6 each group). \*\* P ≤ 0.01 and \*\*\*P ≤ 0.001 compared with control.

Groups	Control	Fructose control	EcN	EcN-2	EcN-3	EcN-4
Hepatic (mg/g Tissue)						
Cholesterol	08.56±2.11	15.38±2.09***	14.08±3.0**	15.67±3.04**	14.22±2.29**	09.37±1.37##
Triglyceride	13.65±1.81	18.45±3.39***	18.02±3.3**	17.45±2.94**	17.99±3.56**	14.78±2.18##

Groups	Control	Fructose control	Ec16	Ec16-2	Ec16-3	Ec16-4
Cholesterol	08.25±1.98	16.55±2.89***	15.21±3.56**	14.67±4.23**	15.01±2.69**	10.01±1.21##
Triglyceride	13.65±2.01	21.34±4.12***	16.99±4.51**	16.99±3.54**	17.54±3.92**	15.55±3.32##

Table 3.4: Hepatic lipid profile. Values are expressed as mean ± SEM (n = 6 each group). \*P ≤ 0.05, \*\* P ≤ 0.01 and \*\*\*P ≤ 0.001 compared with control. ##P ≤ 0.01 compared with fructose control. Hepatic values are expressed as mg/g Tissue.

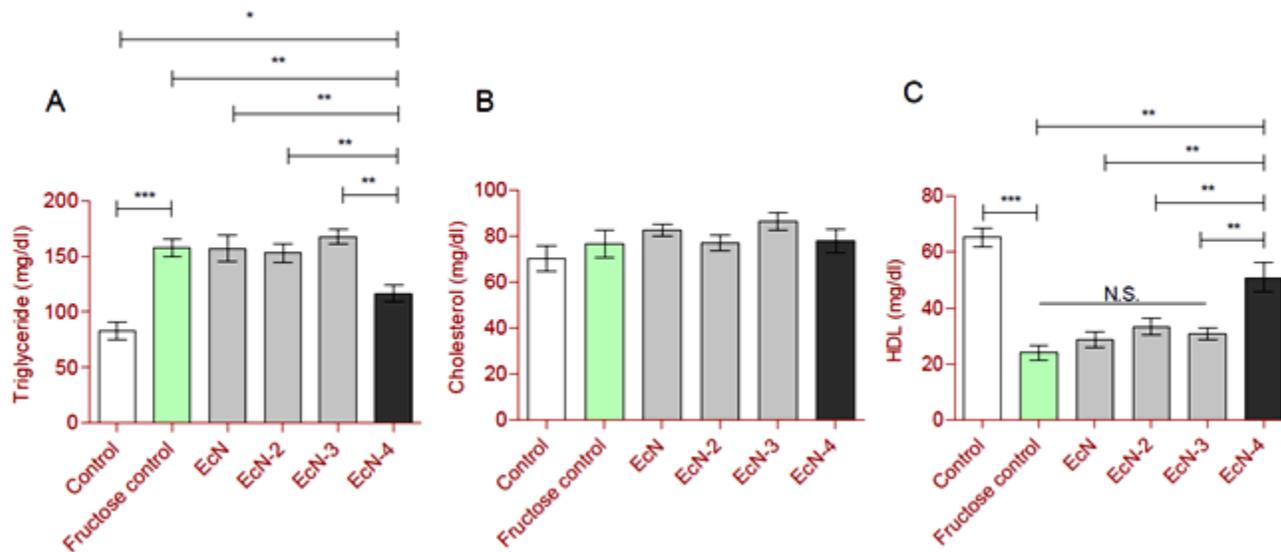


Fig. 3.5: Effect of EcN on serum lipid profile: Values are expressed as Mean  $\pm$  SEM (n= 6 each group). (A) Triglyceride; (B) Cholesterol; (C) HDL. \*P $\leq$  0.05, \*\*P $\leq$  0.01 and \*\*\*P $\leq$  0.001.

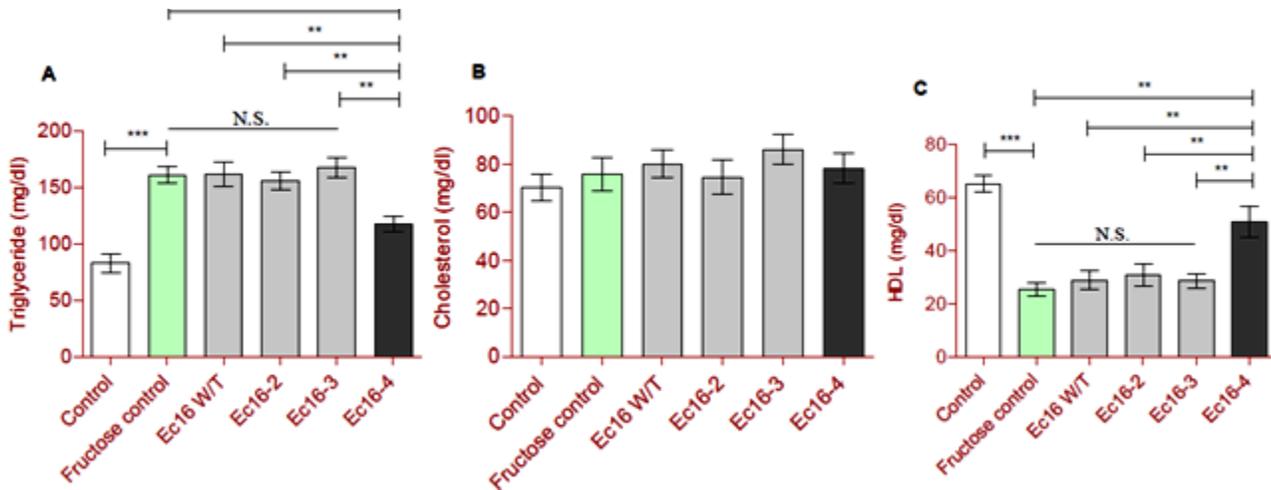
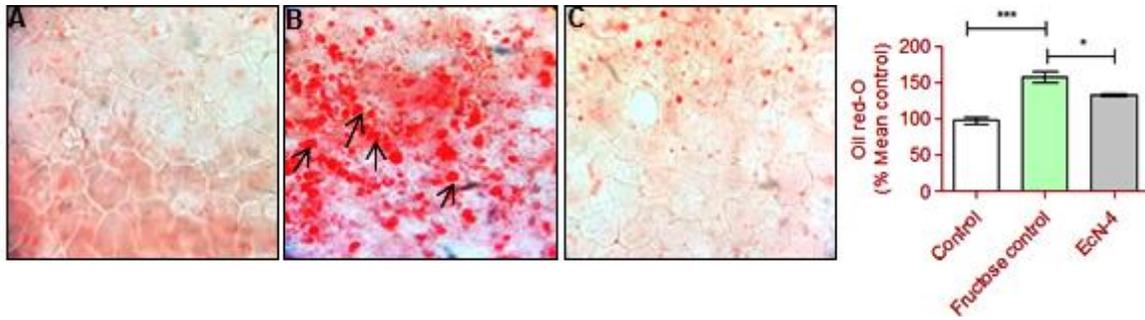
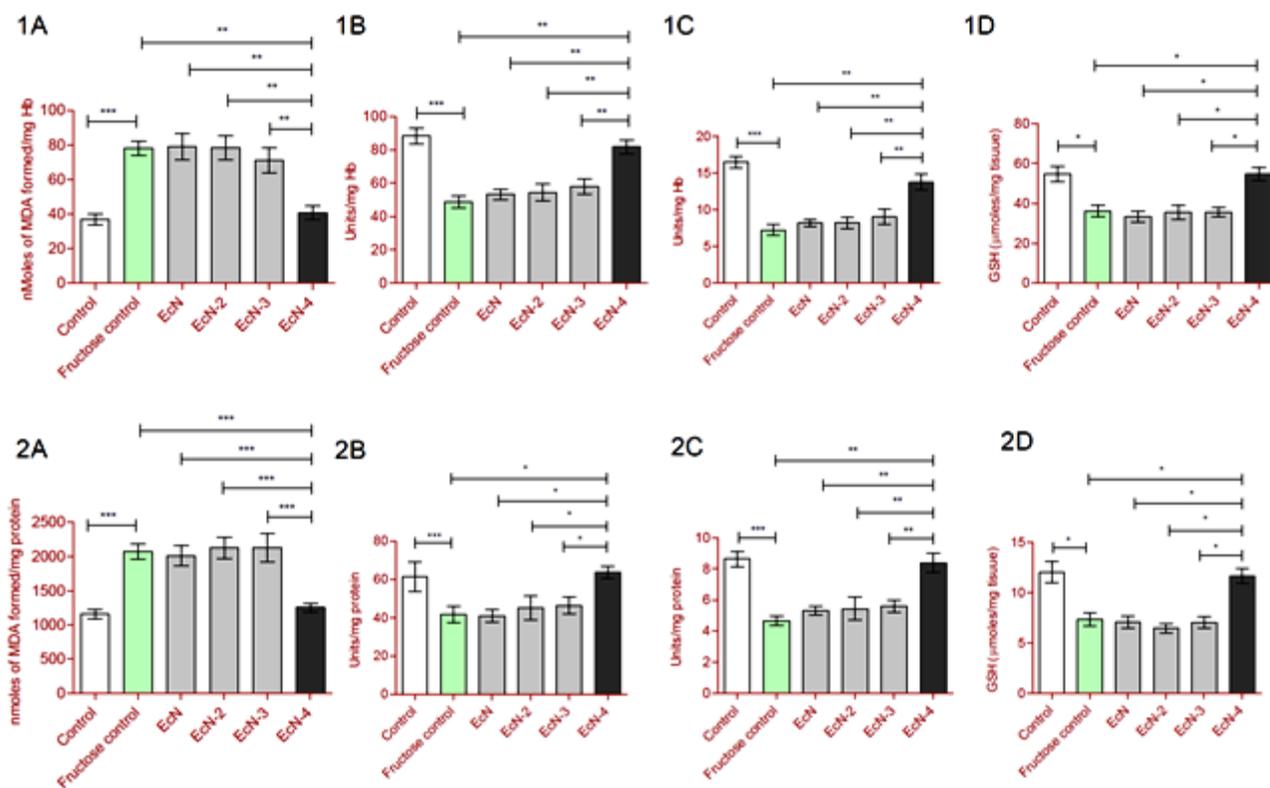


Fig. 3. 6: Effect of Ec16 on serum lipid profile: Values are expressed as Mean  $\pm$  SEM (n= 6 each group). (A) Triglyceride; (B) Cholesterol; (C) HDL. \*\*P $\leq$  0.01 and \*\*\*P $\leq$  0.001.



**Fig 3.7: Oil red O staining of liver tissue. Black arrows indicate the accumulation of lipid droplets in Hepatocytes of fructose fed rat (Fructose control). Images were taken by LEICA DME microscope at 100 X magnification (A) Control, (B) Fructose control and (C) EcN-4. \*P≤ 0.05 and \*\*\*P≤ 0.001**



**Fig. 3.8: Effect of EcN on blood and liver antioxidant status: Values are expressed as Mean  $\pm$  SEM (n= 6 each group). (1A) Blood LPO; (1B) Blood CAT; (1C) Blood SOD; (1D) Blood GSH; (2A) Liver LPO; (2B) Liver CAT; (2C) Liver SOD and (2D) Liver GSH. \*P  $\leq$  0.05, \*P $\leq$  0.01 and \*\*P $\leq$  0.001.**

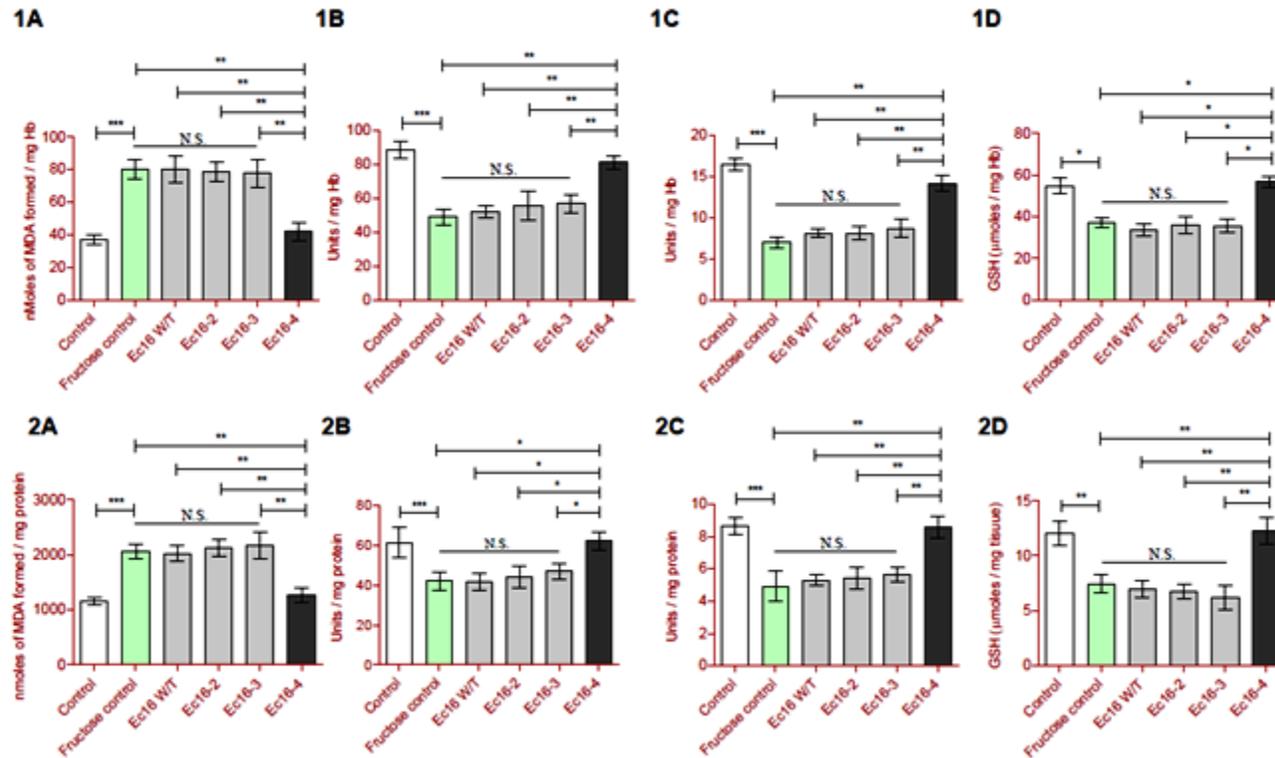
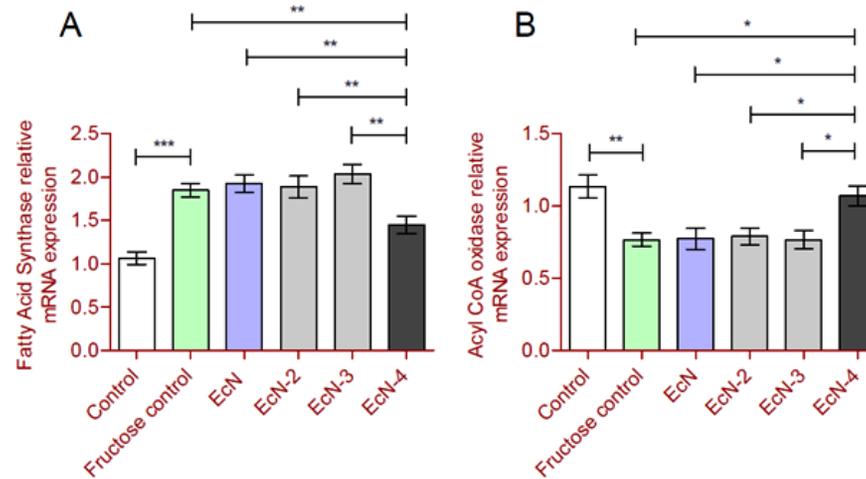
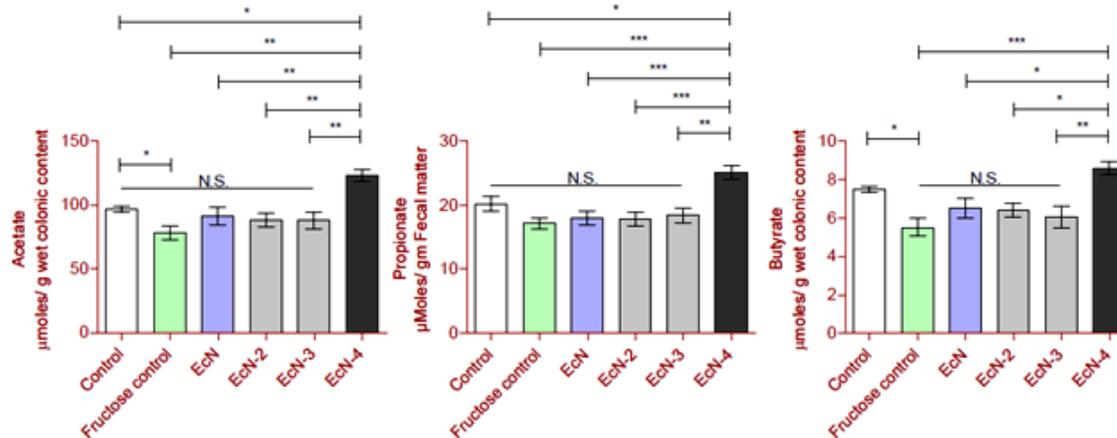


Fig. 3.9: Effect of Ec16 on blood and liver antioxidant status: Values are expressed as Mean  $\pm$  SEM (n= 6 each group). (1A) Blood LPO; (1B) Blood CAT; (1C) Blood SOD; (1D) Blood GSH; (2A) Liver LPO; (2B) Liver CAT; (2C) Liver SOD and (2D) Liver GSH. \*P  $\leq$  0.05, \*P  $\leq$  0.01 and \*\*P  $\leq$  0.001.



**Fig. 3.10:** mRNA expression levels of (A) Fatty acid synthase and Acyl Coenzyme A oxidase in hepatic tissue. Values are expressed as mean  $\pm$  SEM (n=6 each group). \*\*\*P  $\leq$  0.001 \*\*P  $\leq$  0.01 and \*P  $\leq$  0.05.



**Table 3.5:** Colonic short chain fatty acid (SCFA). Values are expressed as mean  $\pm$  SEM (n = 6 each group). \*P  $\leq$  0.05 and\*\* P  $\leq$  0.01 compared with control group. # P  $\leq$  0.05 and ##P  $\leq$  0.001 compared with Fructose control group. Concentration ( $\mu$ Moles/g Fecal matter).

### 3.3 Discussion

Role of gut microbiota in host lipid and glucose metabolism has been recently demonstrated in various studies (**Park et al. 2013**). Probiotics have the capability to manipulate gut microbiota and therefore they might be helpful in preventing risk factors associated with metabolic syndrome including dyslipidemia, elevated fasting glucose levels and insulin resistance. Human and rodents based studies have demonstrated, excessive fat accumulation in liver because of increase in hepatic *de novo* lipid synthesis (**Postic et al. 2008**). Fructose is highly lipogenic sugar as it provides large amount of hepatic triose-phosphate which serves as a precursor for fatty acid synthesis (**Tappy et al. 2010**). Increase in hepatic *de novo* lipogenesis contributes to fructose induced fatty liver and hypertriglyceridemia (**Stanhope et al. 2009**). Recent time studies have focused on various approaches to prevent and/or delay the onset of metabolic complications (**Hsieh et al. 2013**). Foods with low glycemic index and bioactive agents have been predominantly adopted to prevent the delay or onset of disease. Use of single probiotic or consortium of probiotics in fructose induced metabolic effects has been shown to convincingly alleviate metabolic effects; however, these studies were of short term duration (**Yadav et al. 2006; Park et al. 2013**).

Present study involves chronic consumption of fructose for six month duration. Rats fed on fructose diet has been observed with decreased in chow consumption, increased fasting glucose, serum insulin, serum uric acid levels in comparison to rats receiving water (**Nakagawa et al. 2006; Gao et al. 2012**). In this case, oral administration of EcN-4 resulted in reduced fasting glucose, serum insulin and uric acid levels. Modified EcN-4 secretes PQQ in gastrointestinal tract as evident from increases PQQ levels in fecal content and hepatic tissue. Likewise, the reduced serum uric acid levels found in the present study is also as a result of PQQ synthesized from EcN-4. Following this further, oral administration of acetate and propionate to KK-A (y) mice and normal rats reduced glycemia in diabetic hyperglycemic (**den Besten et al. 2013**) Decrease in plasma glucose levels by SCFA may result from increased gut hormones PYY and GLP-1 via activation of the receptors Ffar2 and Ffar3. Moreover, EcN-4 administration also increased SCFA levels in colonic content which may be attributed to the enhanced fermentation by gut microflora utilizing gluconic acid

synthesized by PQQ dependent Glucose dehydrogenase (GDH) of EcN-4. However, this is not the case with EcN as its genome encodes apoGDH gene but does not PQQ biosynthesis genes (**Cress et al. 2013**).

Rats fed on fructose have shown elevated levels of serum triglyceride, a characteristic of metabolic syndrome (**Miller et al. 2008**). PQQ supplementation in diet has been reported to decrease serum triglyceride levels (**Bauerly et al. 2011**). In addition, SCFA has previously been demonstrated to activate fatty acid oxidations, while lipolysis and *de novo* synthesis are inhibited (**den Besten et al. 2013**). This is attributed to reduction in the concentration of free fatty acids in plasma and a decrease in body weight. AMPK activity in liver and muscle tissue has been shown to be increased by SCFAs. AMPK controls the transcriptional activity of several transcription factors such as PPAR $\alpha$ , PPAR $\delta$ , PPAR $\gamma$ , LXR and FXR via activation of PPAR $\gamma$  co-activator (PGC)-1 $\alpha$ . These transcription factors are important in the regulation of cholesterol, lipid and glucose metabolism. As a consequence, *de novo* fatty acid synthesis in the liver is decreased and fatty acid oxidation is enhanced in liver and muscle tissue. In line, administration of MIYAIRI 588—a butyrate-producing probiotic results in decreased serum insulin levels (**Endo et al. 2013**). Finally, decrease of body weight, serum glucose, insulin, uric acid and triglyceride levels in rat group administered with EcN-4 may be a consequence of the combined effect of SCFA produced from gluconic acid and PQQ synthesized by EcN-4. Moreover, similar decrease in hepatic triglyceride levels were also found in rat group administered with EcN-4. This is also supported by the decreased lipid droplet accumulation in hepatic tissue which positively correlates with serum lipid profile. In contrast, groups 3, 4 and 5 did not showed any significant differences in the above parameters suggesting that these effects are outcome of PQQ synthesized by EcN.

Apart from role of PQQ as a co-factor for GDH, it is also a very stable antioxidant molecule (**Rucker et al. 2009**). Studies have investigated antioxidant property of PQQ in murine models and human subjects. Our previous studies demonstrated the efficacy of PQQ as antioxidant molecule in alleviating 1, 2-dimethylhydrazine and ethanol induced toxicity in rat models (**Pandey et al. 2014; Singh et al. 2014**). Present study reinforces the role of PQQ in neutralizing ROS generated by dietary fructose. Maintenance of both systemic and hepatic antioxidant

enzyme activities in EcN-4 rat group can be explained by endogenous delivery of PQQ in gastrointestinal tract and resultant increase in PQQ concentration up to 3 and 2 folds in liver and fecal samples, respectively. Humans depend on plant sources for dietary PQQ. Therefore endogenous production of PQQ produced by EcN-4 is essential for not only in maintaining antioxidant status of the body but also from nutrition prospective (Rucker et al. 2009).

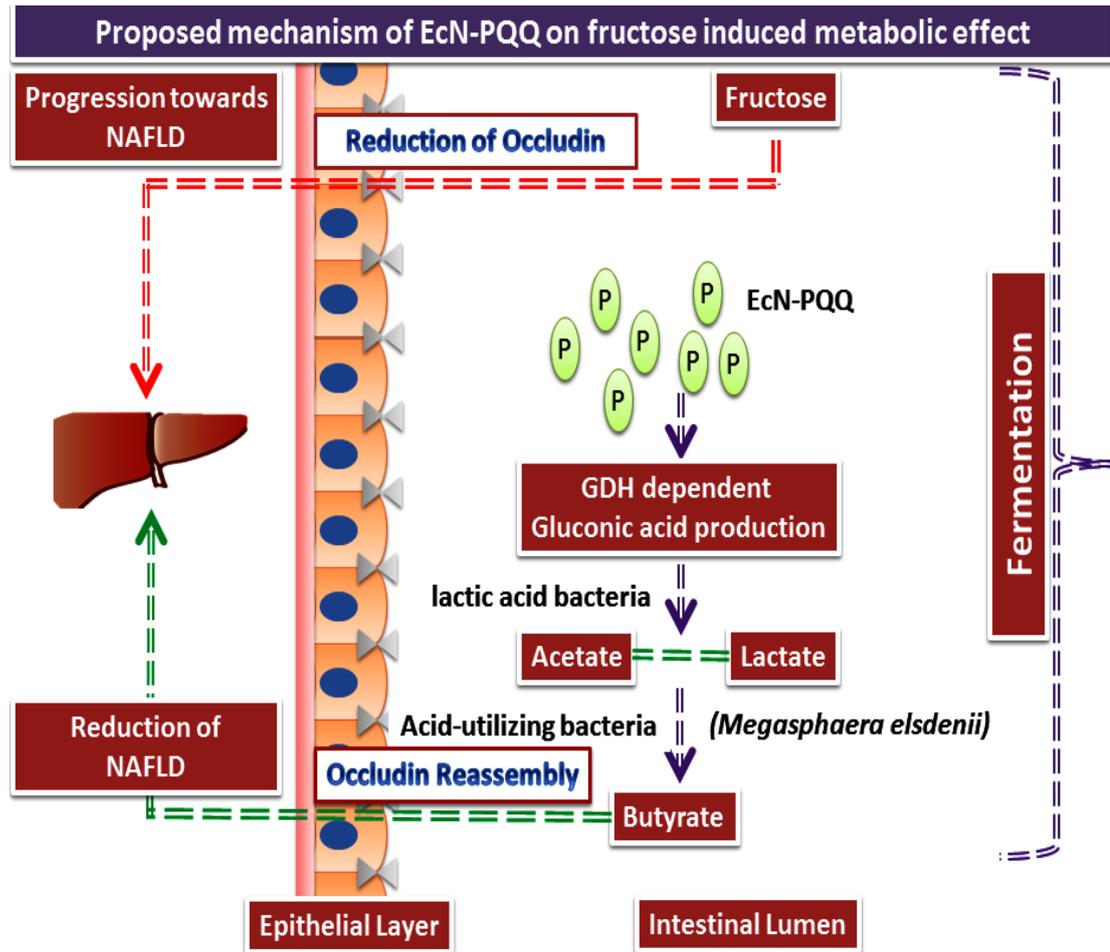


Fig. 3.11 Proposed mechanism of EcN-4 on fructose induced metabolic effects.

Production of SCFA is the result of colonic fermentation and known to confer multiple benefits microbes as well as host (den Besten et al. 2013). In addition to butyrate being used as energy source for colonocytes, SCFA produced in cecum and colon by microbiota can also be detected in peripheral, hepatic and portal blood which in turn can be transported and activate signal transduction in different organs. SCFA levels in rats receiving EcN-4 was found to be higher compared to other rat group receiving EcN, EcN-2 and EcN-3 supplementation. Increase in SCFA content in EcN-4 is because gluconic acid.

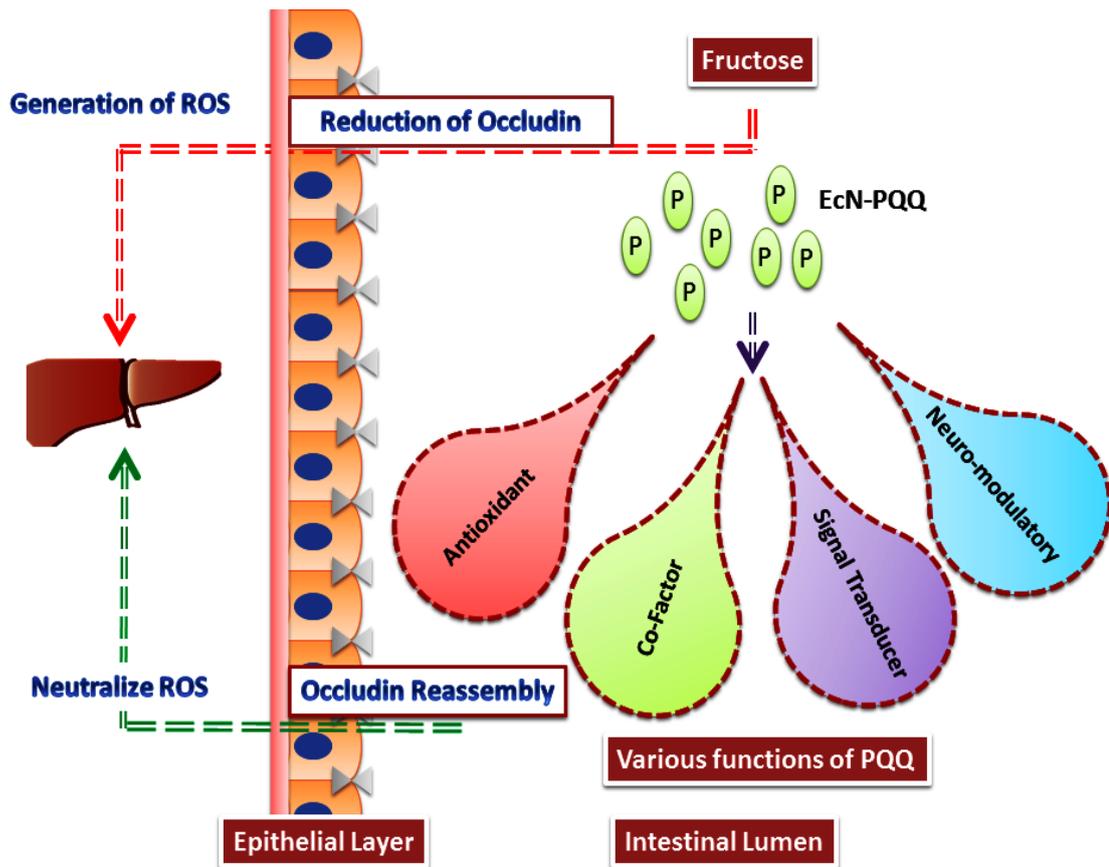


Fig. 3.12 Proposed mechanism of EcN-4 in neutralizing ROS generated by dietary fructose consumption.

The gluconic acid so produced can be metabolized by microflora in lower part of intestine and colon producing SCFA (Kameue et al. 2004). In our earlier studies, EcN-4 ameliorated alcoholic fatty liver disease (AFLD) in rat model while the present study demonstrates its effectiveness in non-alcoholic fatty liver disease (NAFLD). Thus, EcN-4 can serve as natural therapeutic agent for both AFLD and NAFLD. Proposed mechanism of EcN-4 is represented diagrammatically in Fig 3.6 and 3.7.

In conclusion, the physiological beneficial effects observed in the present study are consequence of sustained release of PQQ by EcN-4 and its metabolic product, gluconic acid (prebiotic). The probiotic EcN-4 acts as a symbiotic which suppresses the clinical characteristics of fructose-induced metabolic syndrome, therefore, may provide a natural alternative for the treatment and management of diet-induced metabolic syndrome.