Chapter 4:

Evaluating the effect of probiotic *E. coli* strains harboring plasmid and genomic integrants encoding pyrroloquinoline quinone *(pqq)* and inulosucrase *(inuJ)* gene cluster on sucrose fed Charles Foster rats.

4. Introduction

The prevalence of metabolic disorders has amplified in the present scenario **(Regnault et al., 2013)**. Both, morbidity and mortality, in context to severe cases of metabolic malfunctioning are reported daily in large numbers across the world. This condition has been aided mainly by the surmounting westernized diets, due to cost and production efficiencies. The commonly consumed forms of added-sugars are High Fructose Corn Syrup (HFCS) and sucrose **(Tappy et al., 2010)**. Reports suggest that, in the pre-historic and medieval times, consumption of sucrose rich diets hardly persisted. The hydrolysis of sucrose is mediated by sucrases, a membrane-bound enzyme, present at the brush borders of intestinal epithelium. Accumulating evidences from animal studies supports the fact that fructose component of sucrose contributes substantially in the development of metabolic disorders **(Tappy et al., 2012)**. Consumption of sucrose is associated with hyperlipidemia, hyperuricemia, hypertension, insulin resistance and oxidative stress. Additionally, consumption of added-sugar diets and beverages are associated with hepatic steatosis, independent of the degree of obesity. Accumulation of lipid droplets within hepatocytes along with its inefficient clearance is attributed to chronic liver disease, a condition known as Non-Alcoholic Fatty Liver Disorder (NAFLD). NAFLD mainly involves couple of interrelated 'Hits' on the liver, first hit comprise of hepatic lipid accumulation and hepatic steatosis **(Lim et al., 2010)**. It is accompanied by reduced rate of hepatic lipid clearance by fatty acid catabolism or lipoprotein export of Very Low Density Lipoprotein (VLDL), in comparison to the rate of *de novo* lipogenesis and lipid influx **(Koteish et al., 2001)**. Free fatty acids from adipocytes, which circulate in the liver, also contribute to this hepatic lipid pool. The second hit is inflammation, evidently as a consequence of oxidative stress, lipid peroxidation and cytokine activity. Moreover, it increases free fatty acid influx by TNFα mediated upregulation of hepatic fatty acid translocase attributed to triggering of steatosis **(Ravikumar et al., 2005)**. The VLDL release is microsomal triglyceride transfer protein (MTTP) dependent and via ApoB100. It has been investigated in rodents that long term exposure to fructose inhibits Peroxisomal Proliferation-Activated Receptor-α (PPAR-α) synthesis by unknown mechanism. PPAR-α is required to stimulate MTTP expression. Hence, in absence of PPAR-α, VLDL release from hepatocytes is diminished. In hamster

models, elevated VLDL concentration was observed after administration of high sucrose diet **(Haidari et al., 2002)**. In addition to this, fructose component of sucrose has been proposed to increase citrate availability for lipogenesis by activating Acetyl CoA Carboxylase-1 (ACC1). ACC1 produces malonyl-CoA that inhibits Carnitine Palmitoyl Transferase-1 (CPT1) and thereby reducing ß-oxidation **(Cave et al., 2007)**. Numerous strategies have been opted to delay these effects including drugs and biologics. In addition, herbal plants have also shown promising results in the management of metabolic syndromes including *Cinnamum* extract by improving insulin receptor kinase activity and enhancing insulin sensitivity **(Jarvill et al., 2001)**, fruit of *Gardenia jasminoides*; exhibiting anti-inflammatory activity **(Xiaofeng et al., 2012)** and fruit of *Momordica charantia*, the pericarpium of *Citri reticulate* and L-carnitine; improving NAFLD in female ICR mice **(Leong et al., 2013)**.

PQQ is known to be the third water soluble and heat stable redox cofactor besides, nicotinamides and flavins **(Fetzner et al., 2010)**. It has the ability to catalyze repeated redox cycles (~20, 000 cycles) without being self-oxidized or forming polymers, under appropriate physiological conditions. Female mice and rats fed with PQQ deficient diet have shown reduced fertility and impaired immune response. Moreover, they exhibited 20-80 % reduction in mitochondrial number in liver and altered lipid metabolism. Mitochondrial depletion has been shown to be associated with developmental delays, cardiomyopathy and neuronal dysfunction **(Stites et al., 2006)**. Additionally, PQQ can interact with transcription factors including PGC-1α, NRF and RAS thereby influencing cell proliferation. In earlier Chapter 2, the role of PQQ was demonstrated in alleviating DMH induced oxidative stress and ethanol induced toxicity in our earlier studies.

To circumvent the challenge of metabolic syndromes in recent past, probiotics have been opted as a natural and safe alternative. Moreover, prebiotic like Fructose oligosaccharide (FOS) and Inulin have shown beneficial effect by production of Short Chain Fatty Acids (SCFA). EcN is a gram negative and a well characterized probiotic of *Enterobacteriaceae* family available in the market. It possesses various fitness factors *i.e.* six different iron uptake systems, lack of virulence factor, production of microcin, restoration of damaged epithelium and reduced secretion of pro-inflammatory cytokines. Additionally, it has been shown to trigger higher production of anti-inflammatory cytokines (IL-10, IL-8, and IL-1ß) in Peripheral Blood

Mononuclear Cells (PBMC) compared to *Lactobacillus* and *Bifidobacteria* **(Ghadimi et al., 2008)**. Numerous clinical trials using EcN in human IBD and pouchitis patients have shown convincing remission. Target based development of EcN has been demonstrated in many studies including expression of CAI-1, cyanovirin-N (CV-N), expressing mimic of lacto-N-neotetrose (LNT), expressing PQQ. In the present study, EcN harboring *pqq* and *pqq-inuJ* genes (plasmids and genomic integrants) was investigated for ameliorative effect on sucrose induced metabolic perturbations in rats.

4.1 Materials and method

4.1.1 Experimental design (Sucrose-plasmid based)

For this study rats were divided into seven different groups (n=6) as follows: Group I received pellet diet and served as control group, Group II received pellet diet and 20 % sucrose in drinking, Group III received pellet diet, 20 % sucrose in drinking water and EcN, Group IV received pellet diet, 20 % sucrose in drinking water and EcN-2, Group V received pellet diet, 20 % sucrose in drinking water and EcN-6, Group VI received pellet diet, 20 % sucrose in drinking water and EcN-7 and Group VII received pellet diet, 20 % sucrose in drinking water and EcN-8 **(Fig. 4.1)**. Probiotics (10^9 *cfu*) was supplemented to all groups except Group 1 and 2 once per week till six months. All the plasmids constructs and strains used in this study are listed in **Table 4.1.**

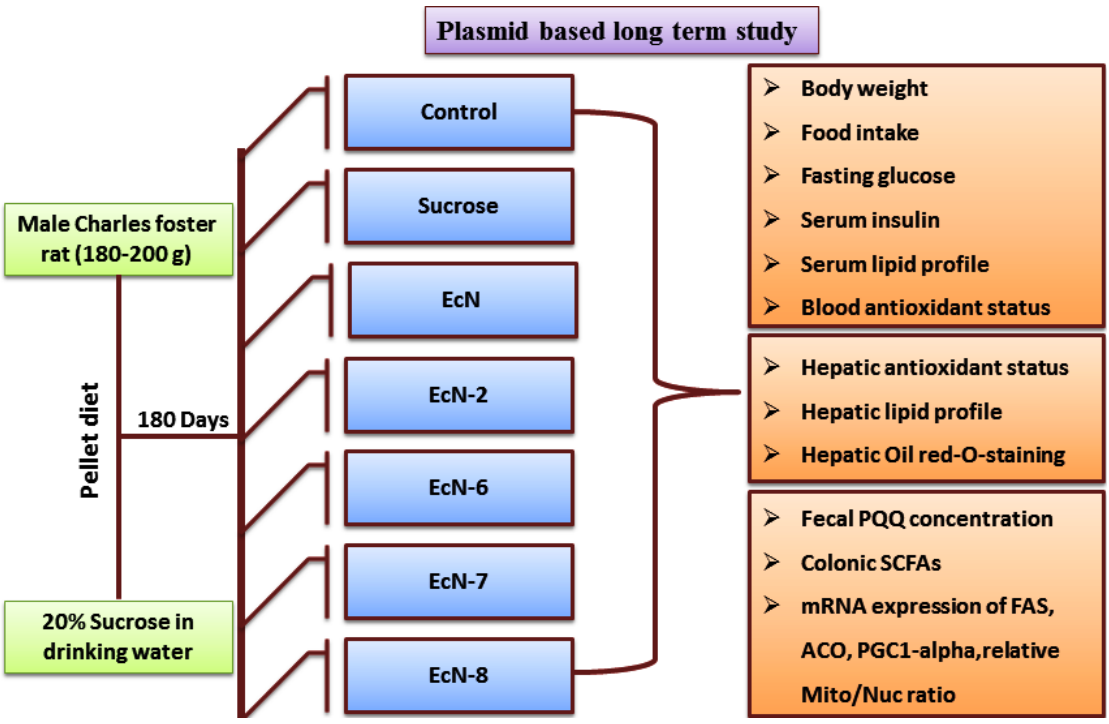


Fig. 4.1 Work plan for the plasmid based long term animal experimentation on sucrose fed rats.

Plasmids	Characteristics	References
pMAL_P2	Periplasmic expression vector	Michael et al.,1998
6a (pMAL_P2 Δ *Lac*^{Iq})	pMAL_P2 vector with deleted *Lac*^{iq} (repressor)	Unpublished previous lab work
6a-*pqq*-1	pMAL_P2 Δ *Lac*^{Iq} with *pqqABCDE* (Xba I site)	This study
6a-*pqq*-2	pMAL_P2 Δ *Lac*^{Iq} with *pqqABCDE* (*Bgl* II-*Xba* I sites)	This study
6a-*pqq*-2-*inuJ*	6a-*pqq*-2 with *inuJ*	This study
pGRG_36-*vgp*	pGRG_36 integration vector harboring *vgb*, *gfp* and *pqqABCDE* genes	This study
pGRG_36-*vgp-inuJ*	pGRG_36 integration vector harboring *vgb*, *gfp*, *pqqABCDE* and *inuJ* genes	This study
Strains	**Characteristics**	**References**
EcN	*Escherichia coli* Nissle 1917 (EcN) (Probiotic strain)	Sonnenborn and Schulze (2009)
EcN-2	EcN strain with genomic integration of *vgb* and *gfp* genes.	Singh et al., 2014
EcN-5	EcN-2 with pTPQ-1 plasmid	Singh et al., 2015
EcN-6	EcN-2 with 6a (pMALp-2 Δ *lacI*^{q}) plasmid	This study
EcN-7	EcN-2 harboring 6a-*pqq*-2 (*Bgl*II/*Xba*I site) plasmid	This study
EcN-8	EcN-2 harboring 6a-*pqq*-2-*inuJ* plasmid	This study
EcN-9	EcN strain with genomic integration of *vgb*, *gfp* and *pqqABCDE* genes (using pGRG_36-*vgp* plasmid)	This study
EcN-10	EcN strain with genomic integration of *vgb*, *gfp*, *pqqABCDE* and *inuJ* genes (using pGRG_36-*vgp-inuJ* plasmid)	This study

Table 4. 1 Plasmid constructs and strains used in the study

4.1.2 Experimental design (Sucrose-Genomic integrants)

For this study rats were divided into seven different groups (n=6) as follows: Group I received modified diet and served as control group, Group II received modified diet and 20 % sucrose in drinking, Group III received modified diet, 20 % sucrose in drinking water and EcN-2, Group IV received modified diet, 20 % sucrose in drinking water and EcN-7, Group V received modified diet, 20 % sucrose in drinking water and EcN-8, Group VI received modified diet, 20 % sucrose in drinking water and EcN-9 and Group VII received modified diet, 20 % sucrose in drinking water and EcN-10 **(Fig. 4.2)**. Probiotics (10^9 *cfu*) was supplemented to all groups except Group 1 and 2 once per week till six months. All the plasmids constructs and strains used in this study are listed in **Table 4.1.**

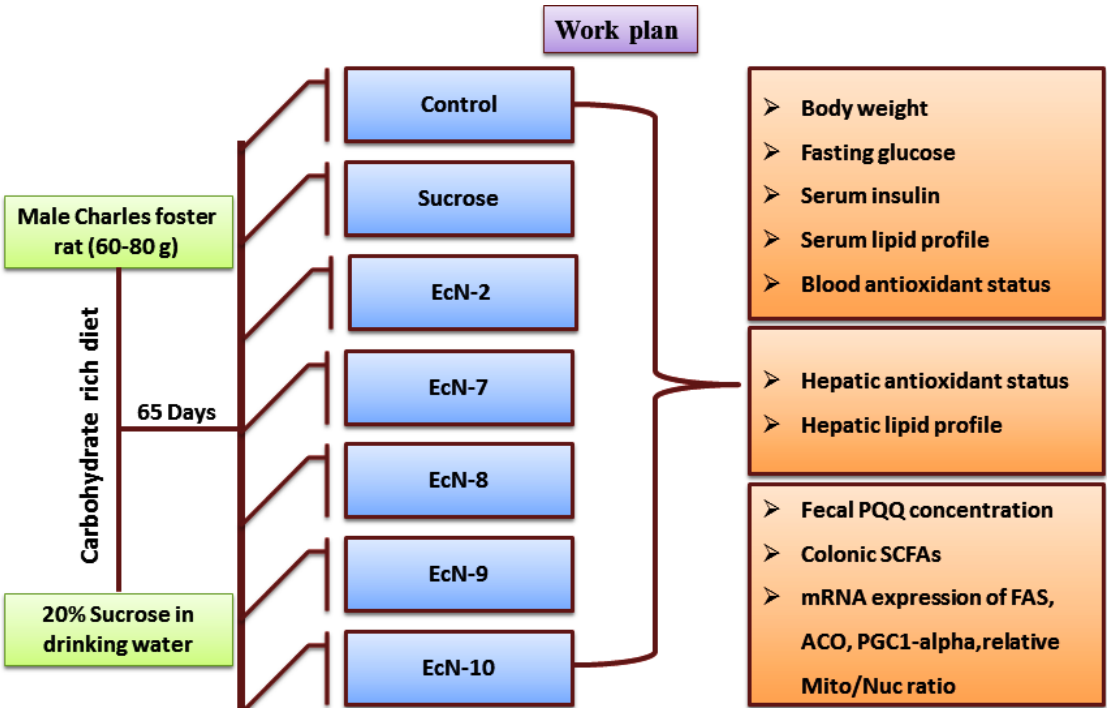


Fig. 4.2 Work plan to test EcN plasmid *vs* genomic integrants on sucrose induced rat models.

4.2 Results (Sucrose plasmid based)

4.2.1 Sub-cloning, confirmation and functional characterization of EcN-7 and EcN-8

Strategy used for construction of recombinant plasmid pMALp2Δ*lacIq-inuJ-pqq-2* is represented in **Fig. 4.3.** PCR amplification of *pqqABCDE* gene was carried out from TA cloning vector followed by sub-cloning in *Xba*I site of pMALp2Δ*lacIq-inuJ* plasmid. Authentication of recombinant plasmid (pMALp2Δ*lacIq-inuJ-pqq-2*) was carried out by PCR amplification and restriction digestion **(Fig 4.4 and 4.5)**. Functionality of *pqq* and *inuJ* gene was confirmed on Tris Buffered medium and sucrose containing minimal M-9 medium **(Fig 4.6)**. This recombinant plasmid was used to transform EcN-2.

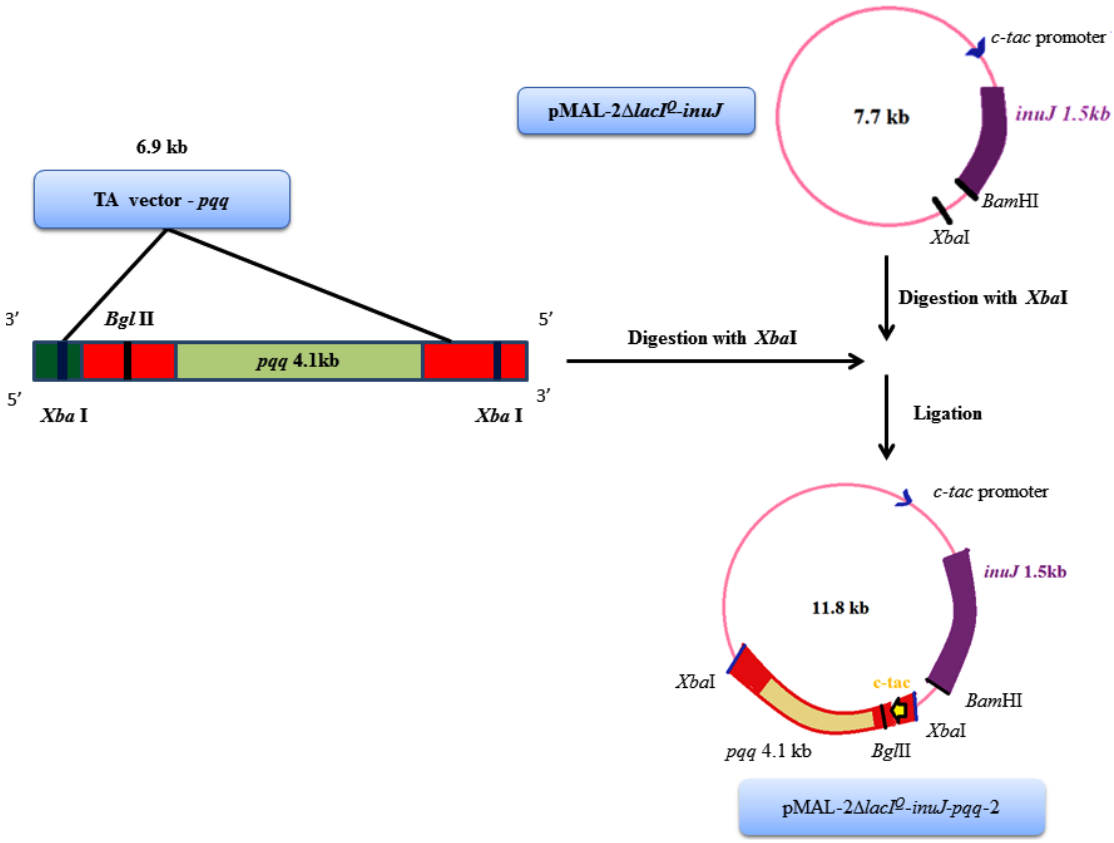
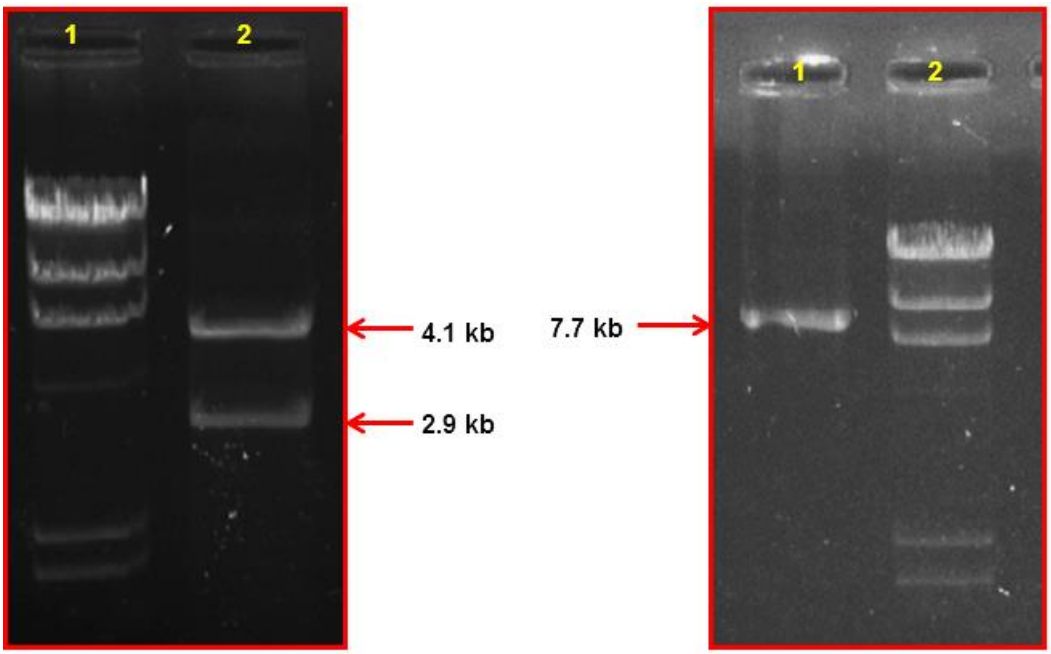


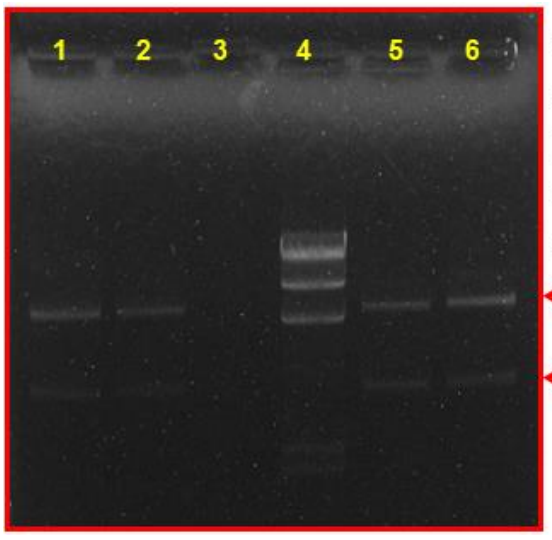
Fig. 4.3 Strategy used for construction of recombinant plasmid pMALp2Δ*lacIq-inuJ-pqqABCDE*.



Lane 1: λ DNA *Hind* III Marker
Lane 2: T-PQQ digest with *Xba*I

Lane 1: λ DNA *Hind* III Marker
Lane 2: pMALp2 Δ lac Iq *inuJ Bam* HI digest

Fig. 4.4 Authentication of recombinant plasmids containing *ppq* gene cluster in T-vector and *inuJ* in pMALP2Δ*lacIq* vector.



Lane 1: pMALp2 Δ *lac Iq inuJ-pqq -2 Xba*I digest
Lane 2: pMALp2 Δ *lac Iq inuJ-pqq -2 Xba*I digest
Lane 4: λ DNA *Hind* III Marker
Lane 5: pMALp2 Δ *lac Iq inuJ-pqq-2 Xba*I digest
Lane 6: pMALp2 Δ *lac Iq inuJ-pqq -2 Xba*I digest

Fig. 4.5 Restriction digestion of pMaL-P2 Δ *lacIq pqq-2-inuJ* with *Xba*I.

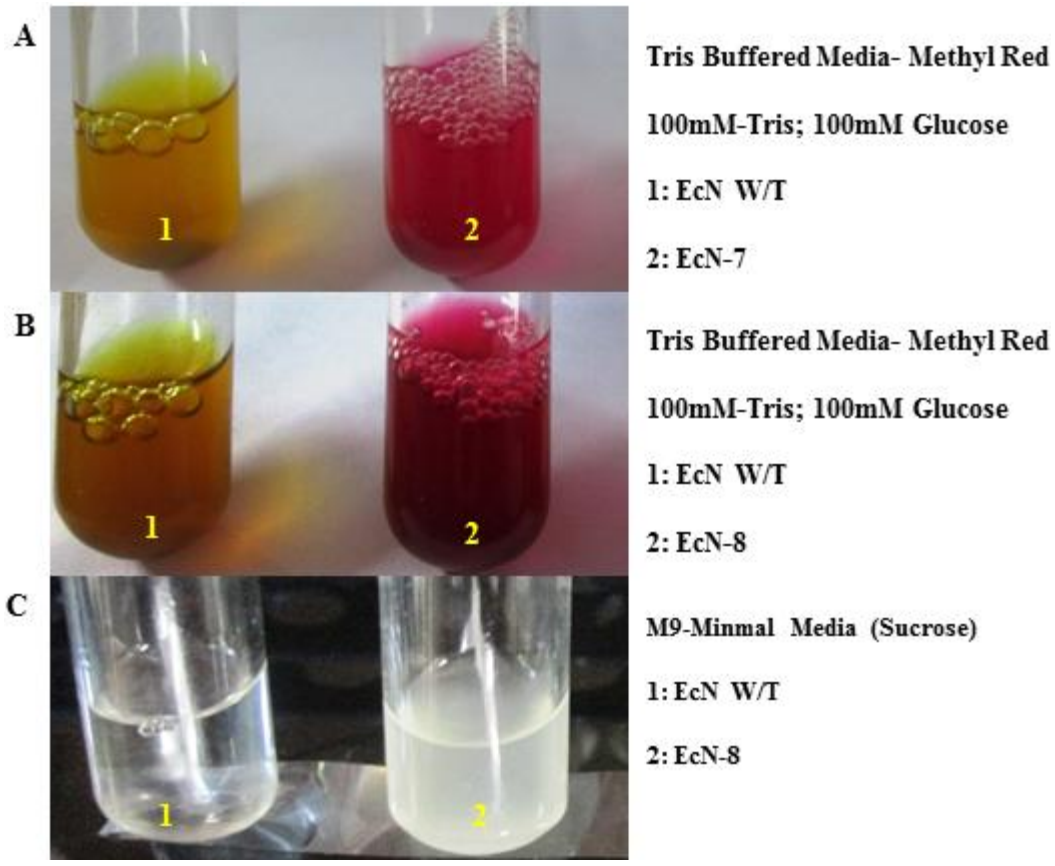


Fig. 4.6 Functional characterization of EcN-7 and EcN-8 on Tris buffered minimal medium and sucrose containing minimal M9 medium.

4.2.2 Effect of modified EcN on Body weight, food intake, glucose and serum insulin levels

Sucrose (20 % in drinking water) feeding resulted in body weight gain in group 2 rats on comparison with group 1 rats **(Fig. 4.7 A)**. Rats receiving EcN-7 and EcN-8 treatment exhibited reduction in body weight, with maximum effect in EcN-8 treated group. However, group administered with EcN-1, 2 and 6 displayed no significant differences. Food intake of all the groups was similar except in group 1 rats **(Fig. 4.7 B)**. Fasting serum glucose levels exhibited marked increase in group 2 rats receiving only sucrose without probiotic supplementation in comparison to group 1 **(Fig. 4.7 C)**. Supplementation of EcN-7 and EcN-8 restricted these levels close to normal value. Here also, EcN-8 treatment was more efficient than EcN-7. As observed in case of body weight, all the other groups did not show any significant differences in fasting serum glucose levels.

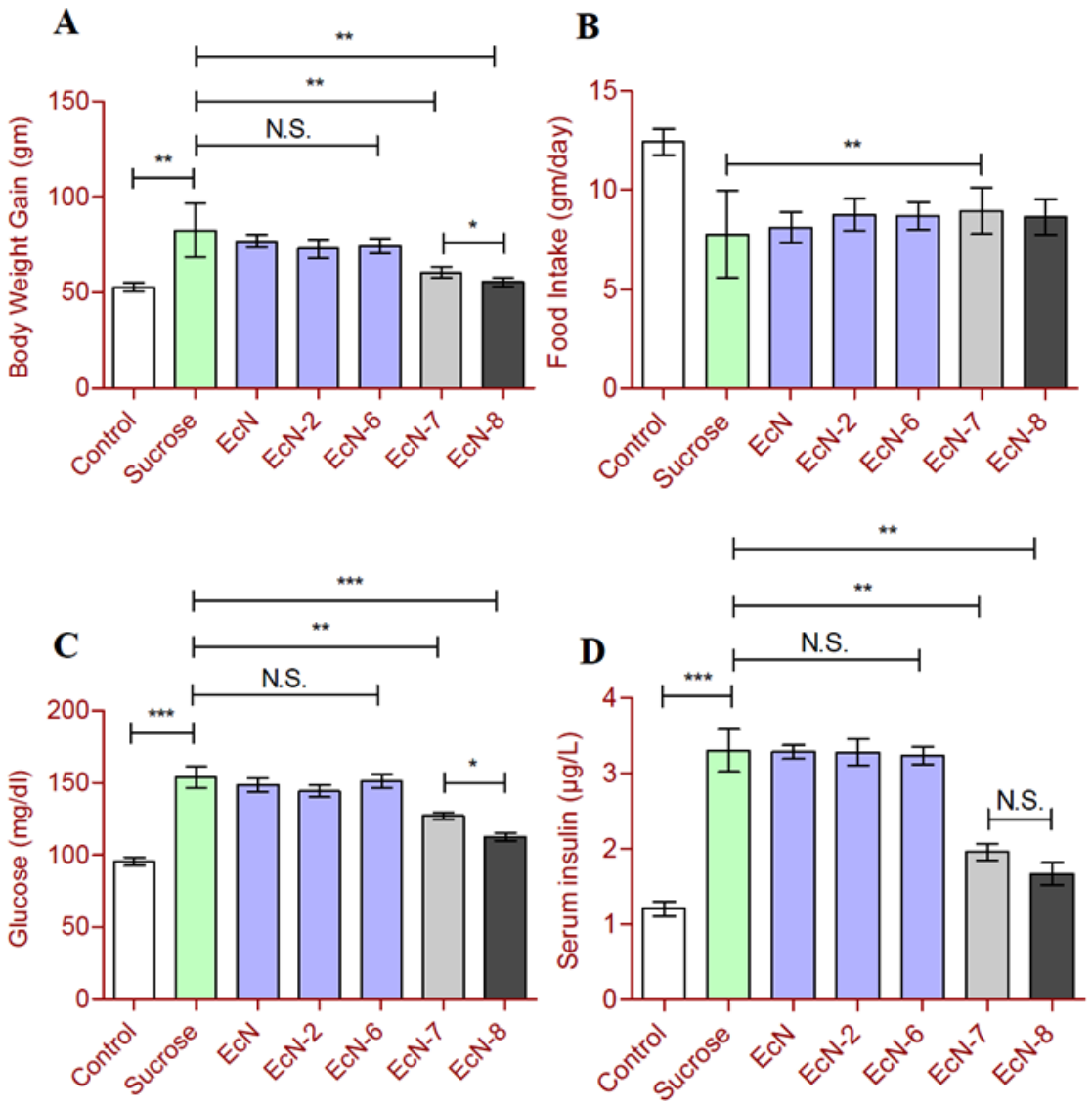


Fig. 4.7 Effect of genetically engineered probiotic *E. coli* Nissle 1917 on (A) Body weight gain (gm) (B) Food intake gm/day (C) Fasting Glucose levels (mg/dl) and (D) serum insulin (µg/L) of sucrose fed rats. Values are expressed as mean ± SEM (n=6 each group). ** $P \leq 0.01$ and *** $P \leq 0.001$.

Elevated insulin levels, one of the characteristic of metabolic syndrome, was observed in group 2 rats **(Fig. 4.7 D)**. Wild type EcN, EcN-2 and EcN-6 had no effect on high serum insulin levels. However, EcN-7 treatment maintained lower serum insulin levels with maximum effect observed in EcN-8 administered group.

4.2.3 Effect of modified EcN on serum and hepatic triglyceride levels

Elevated serum triglyceride levels, characteristic of metabolic syndrome exhibited almost 2 fold increase in group 2 rats in comparison to group 1 rats **(Fig. 4.8)**. Supplementation with EcN-7 and EcN-8 restricted these levels within normal

range. Interestingly, EcN secreting both PQQ and InuJ was most effective. Similar results were also observed in case of serum cholesterol and HDL levels. However, EcN-1, 2 and 6 had no significant effect on these parameters.

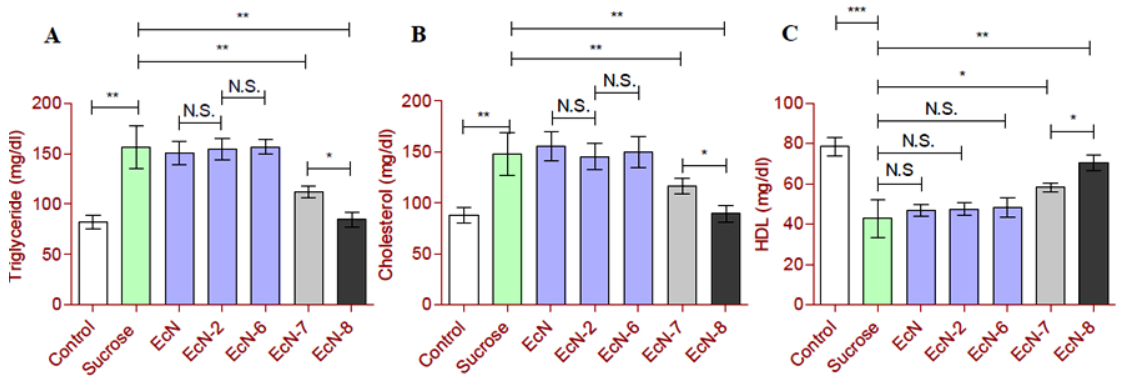


Fig. 4.8 Effect of genetically engineered probiotic *E. coli* Nissle 1917 on serum lipid profile of rats. (A) Triglyceride (B) Cholesterol and (C) HDL. Values are expressed as mean ± SEM (n=6 each group).*P ≤ 0.05 , ** P ≤ 0.01 and ***P≤ 0.001. Values are expressed as mg/dl.

To further support serum lipid profile finding, hepatic lipid profile was monitored. Serum lipid profile in group 2 rats also exhibited elevated cholesterol and triglyceride levels compared with group 1. Treatment with EcN-7 and EcN-8 reflected decrease in these levels. All other groups including EcN-1, 2 and 6 had no change in these parameters **(Table 4.2)**.

Groups	Control	Sucrose	EcN-1	EcN-2	EcN-6	EcN-7	EcN-8
Cholesterol (mg/g Tissue)	09.21± 1.98	16.23± 3.55**	15.88± 4.02**	14.92± 4.45**	15.32± 3.53**	11.21± 2.45##	09.92± 2.34##
Triglyceride (mg/g Tissue)	12.65± 2.11	17.56± 3.19**	17.82± 3.42**	18.04± 3.63**	17.82± 3.99**	15.87± 3.76##	15.54± 3.87##

Table 4.2 Effect of genetically engineered probiotic *E. coli* Nissle 1917 on liver lipid profile of rats. Values are expressed as mean ± SEM (n=6 each group). ** P ≤ 0.01 compared with control. ##P ≤ 0.01 compared with Sucrose control. Values are expressed as mg/g Tissue.

4.2.4 Effect of modified EcN on blood and liver antioxidant status

Fructose component of sucrose is solely metabolized in liver resulting in oxidative stress. To investigate antioxidant potential of PQQ and PQQ-InuJ secreting EcN, blood and liver antioxidant status were monitored **(Fig. 4.9)**. Consumption of sucrose resulted in marked increase in LPO and reduced GSH levels in comparison to

group 1 rats. Moreover, antioxidant enzyme activity (SOD and CAT) were also found to be decreased in this group. Treatment with EcN-7 and EcN-8 decreased oxidative stress correlated with increased GSH and antioxidant enzyme activities with no significant difference between these two groups. All other groups, EcN-1, 2 and 6 did not exhibit any significant effect on these parameters. Similar results were also found in case of hepatic antioxidant status **(Fig. 4.10)**.

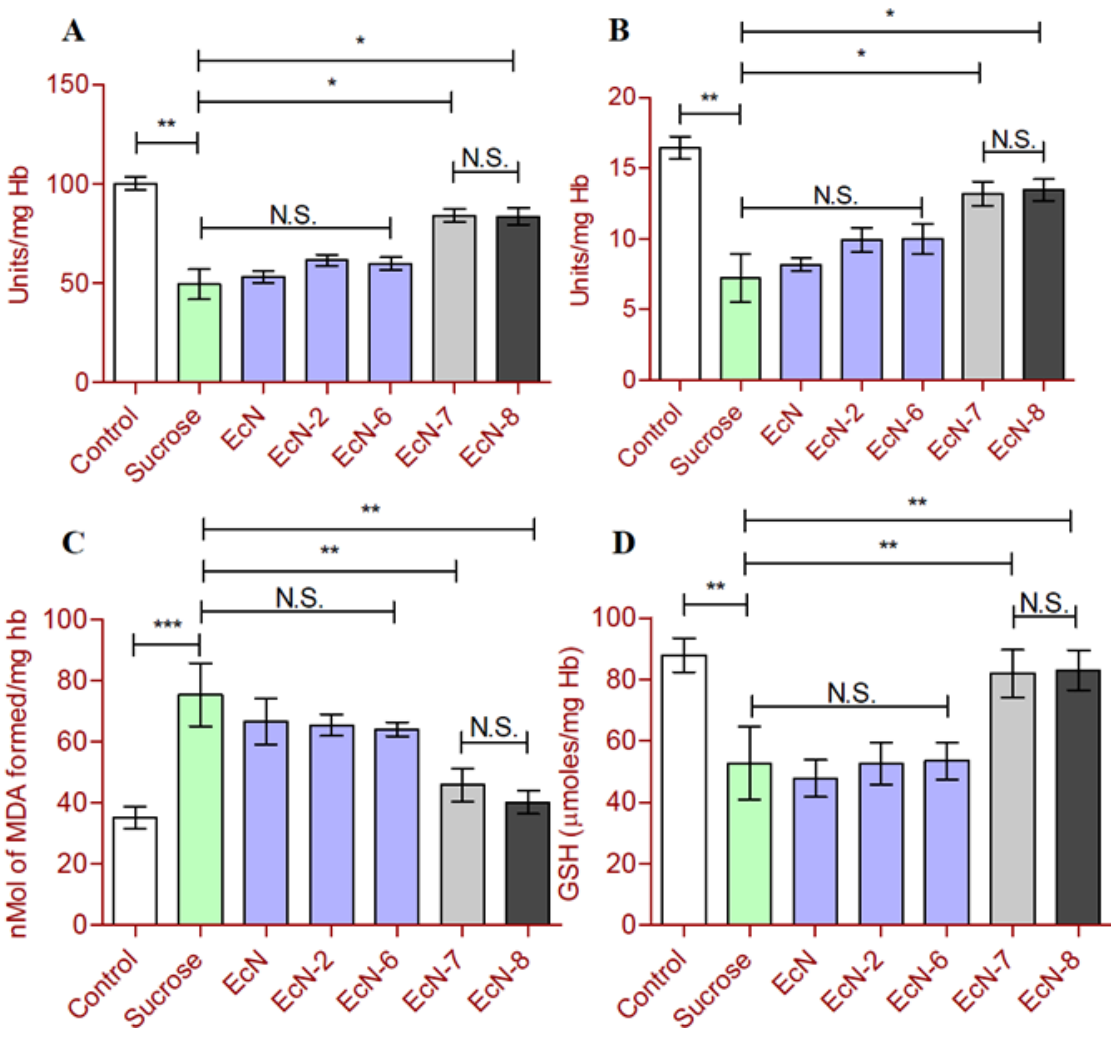


Fig. 4.9 Effect of genetically engineered probiotic *E. coli* Nissle 1917 on Blood (A) Catalase (CAT) activity (B) Superoxide Dismutase (SOD) activity, (C) RBC Lipid peroxidation (LPO) and GSH levels. Values are expressed as mean ± SEM (n=5 each group). *P ≤ 0.05, **P ≤ 0.01 and *** P ≤ 0.001.

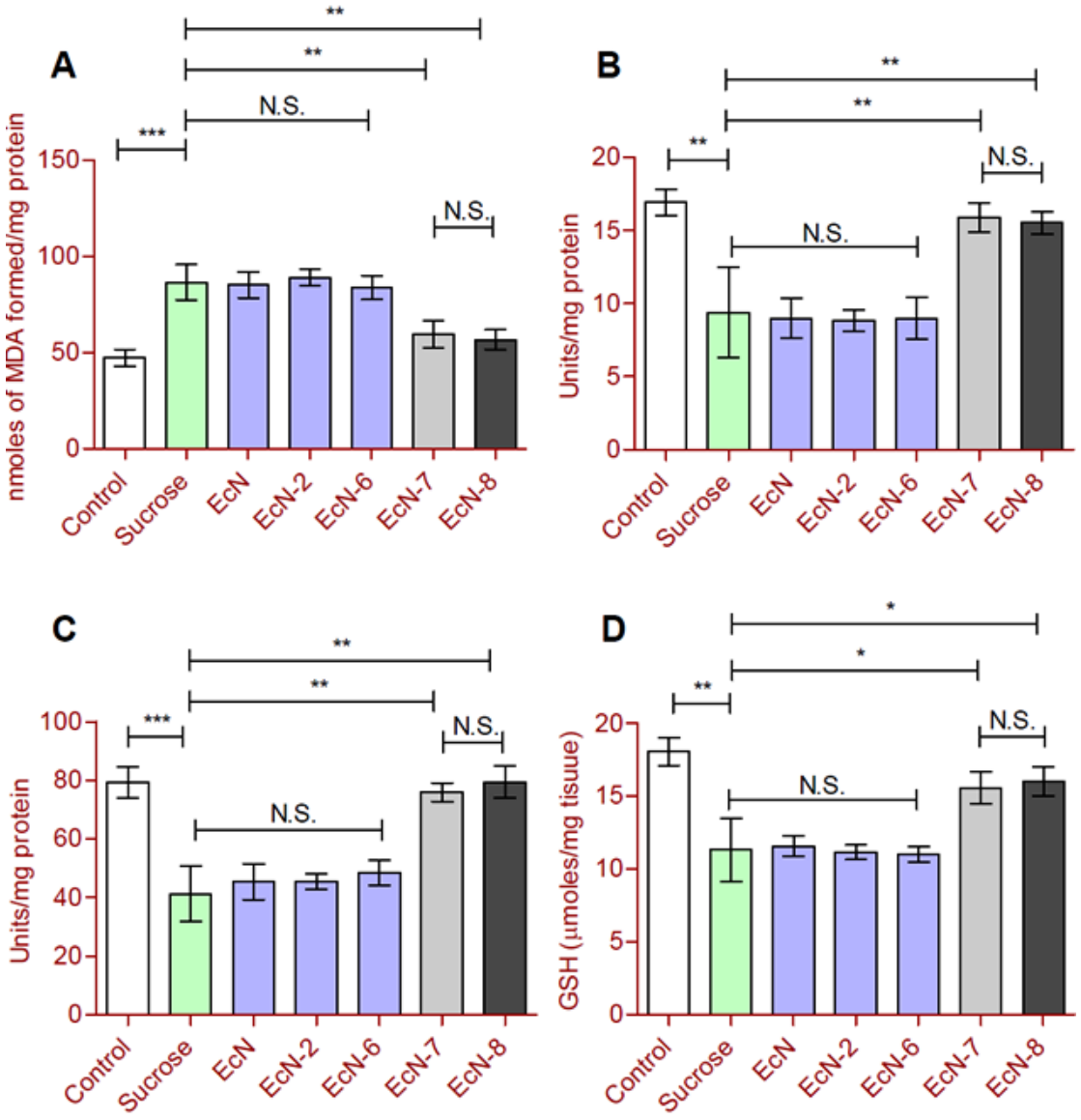


Fig. 4.10 Effect of genetically engineered probiotic *E. coli* Nissle 1917 on liver (A) Lipid peroxidation (LPO) (B) Superoxide Dismutase (SOD) (C) Catalase (CAT) and (D) GSH levels. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, **P ≤ 0.01 and *** P ≤ 0.001.

4.2.5 Effect of modified EcN on colonic short chain fatty acid (SCFA)

In case of group receiving EcN-8 two prebiotic molecules are formed; first gluconic acid and secondly, metabolism of sucrose by InuJ enzyme converts fructose moiety of sucrose into fructose oligosaccharide (FOS). Both gluconic acid and FOS so formed move to lower part of gastrointestinal tract by intestinal motility and is metabolized mostly by *Bifidobacterium* resulting in SCFA formation as end product. Group receiving EcN-7 and EcN-8 showed marked increase in colonic SCFAs (Acetate,

Propionate and Butyrate) content with maximum production in EcN-8 treated group in comparison to control group **(Table 4.3)**.

Group	Total (SCFA)	Acetate	propionate	Butyrate
Control	122.93 ± 9.25	91.71 ± 4.9	22.871 ± 3.10	8.35 ± 1.149
Sucrose control	101.81 ± 12.0**	76.74 ± 7.5**	19.174 ± 3.09*	5.89 ± 1.44**
EcN-7	132.28 ± 12.76	100.06 ± 6.5	22.79 ± 3.05	9.42 ± 3.31
EcN-8	175.94 ± 18.30***	139.55 ± 11.93***	34.31 ± 4.29**	11.87 ± 2.07*

Table 4.3 Effect of genetically modified probiotic *E. coli* Nissle 1917 on Colonic SCFA. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, ** P ≤ 0.01 and* P ≤ 0.001 compared with control group. Concentration (µMoles/ gm Fecal matter).**

4.2.6 Effect of modified EcN on mRNA expression profile of FAS, ACoX, PGC-1α and Mit/Nuc DNA ratio.

Hepatic fatty acid synthase (FAS) gene expression was found to be elevated in groups 2 rats in comparison to group 1 rats. Administration of EcN-7 and 8 decreased FAS gene expression with maximum effect observed in group 8 rats. Similarly, Acyl CoA oxidase (ACoX) gene expression exhibited marked increase in EcN-7 and EcN-8 administered rats. However, EcN-2 administration did not show any effect. PGC-1α and mitochondrial / nuclear expression was found to be higher in EcN-7 and 8 receiving rats. However, there was no significant difference between EcN-7 and EcN-8 fed rats. As observed in case of FAS and ACoX, EcN-2 did not had any effect on PGC-1α and mitochondrial / nuclear expression.

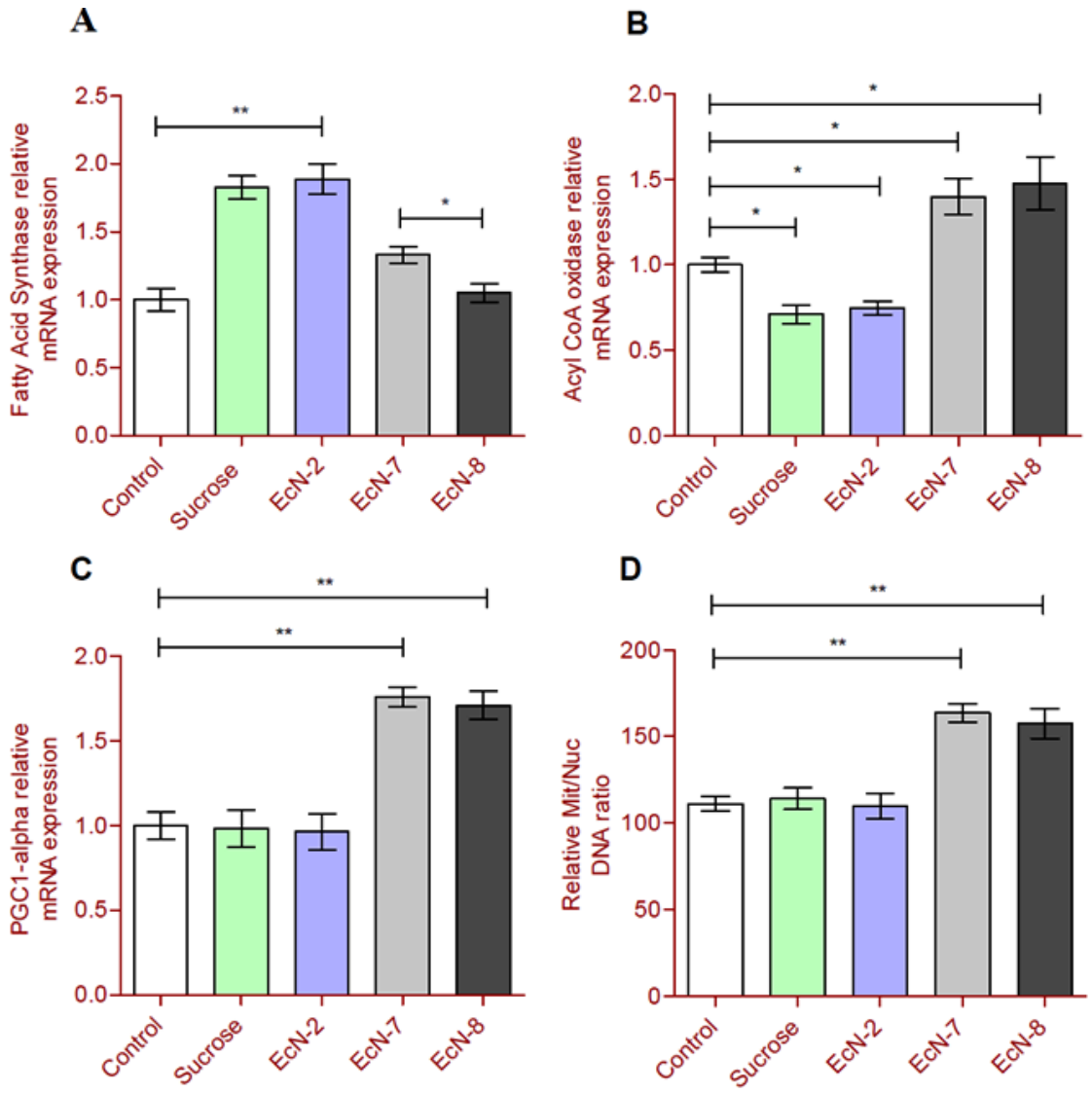


Fig. 4.11 Effect of genetically modified *E. coli* Nissle 1917 on liver (A) Fatty acid synthase, (B) Acyl CoA oxidase, (C), PGC1-alpha relative mRNA expression and relative mitochondria to nucleus DNA ratio. Values are expressed as mean ± SEM (n=5 each group). *P ≤ 0.05 and P ≤ 0.01 compared with control group.**

4.3 Result (Sucrose genomic integrants)

4.3.1 Genomic integration and characterization of EcN:: *vgb-gfp-pqq* and EcN:: *vgb-gfp-pqq-inuJ*

PCR amplification of *pqqABCDE-inuJ* from pMALp2Δ*lacIq-inuJ-pqqABCDE* plasmid was carried out using Forward primer: TGACAATTAATCATCGGCTCGTATAATGGATCGAA TTGTGAG and Reverse primer: CGCTTCTGCGTTCTGATTTAATCTGTATCAGG by gradient PCR **(Fig. 4.12)**. Amplified DNA fragment containing *pqqABCDE-inuJ* was sub-cloned at *Not*1 site followed by end filled in pGRG36 *vgb-gfp* plasmid **(Fig. 4.13)**. pGRG36 *vgb-gfp-pqqABCDE-inuJ* recombinant plasmid was confirmed by restriction digestion **(Fig. 4.14).** pGRG36 *vgb-gfp-pqqABCDE-inuJ* was used to transform EcN and selection of positive clones were carried on ampicillin containing LA agar plate. Genomic integration was carried out by Tn7 mediated genomic integration according to protocol of **(Crieg et al., 2006).** Genomic integrants of EcN::*vgb-gfp-inuJ-pqqABCDE* (EcN-10) were selected on non-ampicillin containing plate as 42 ^{0}C and confirmed by PCR amplification of the *vgb* gene and *inuJ-pqqABCDE* gene cluster resulting in an amplicon sizes of 714 bp and 7.16 kb, respectively **(Fig. 4.15)**.

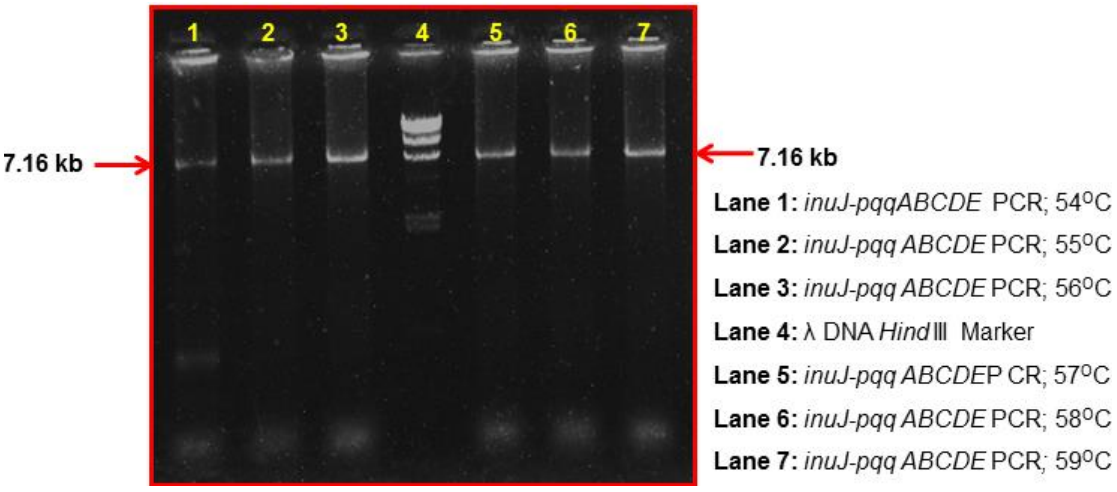


Fig. 4.12 Gradient PCR of *pqqABCDE-inuJ* amplicon from pMALp2Δ*lacIq-inuJ-pqqABCDE* recombinant plasmid.

Functionality of EcN-9 and EcN-10 was tested by growing them on Tris buffered medium containing methyl red and minimal medium containing sucrose as carbon source.

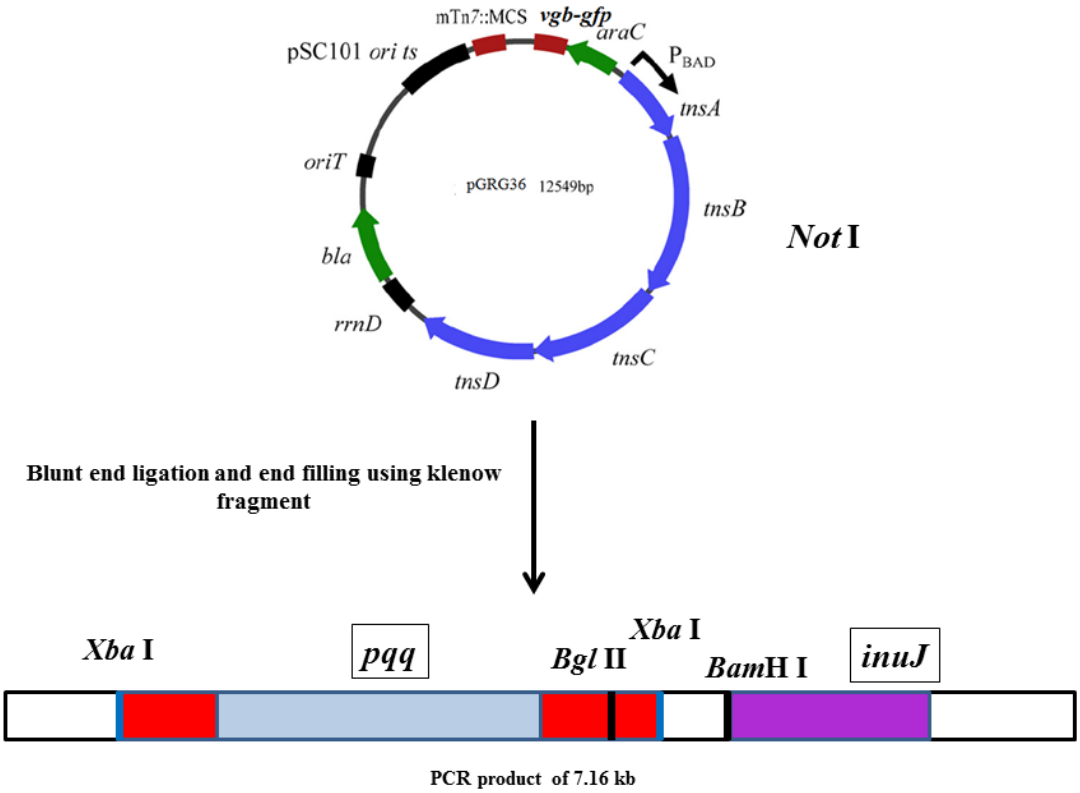


Fig. 4.13 Strategy used for sub-cloning of *pqq-inuJ* amplicon in pGRG36 *vgb-gfp* recombinant plasmid.

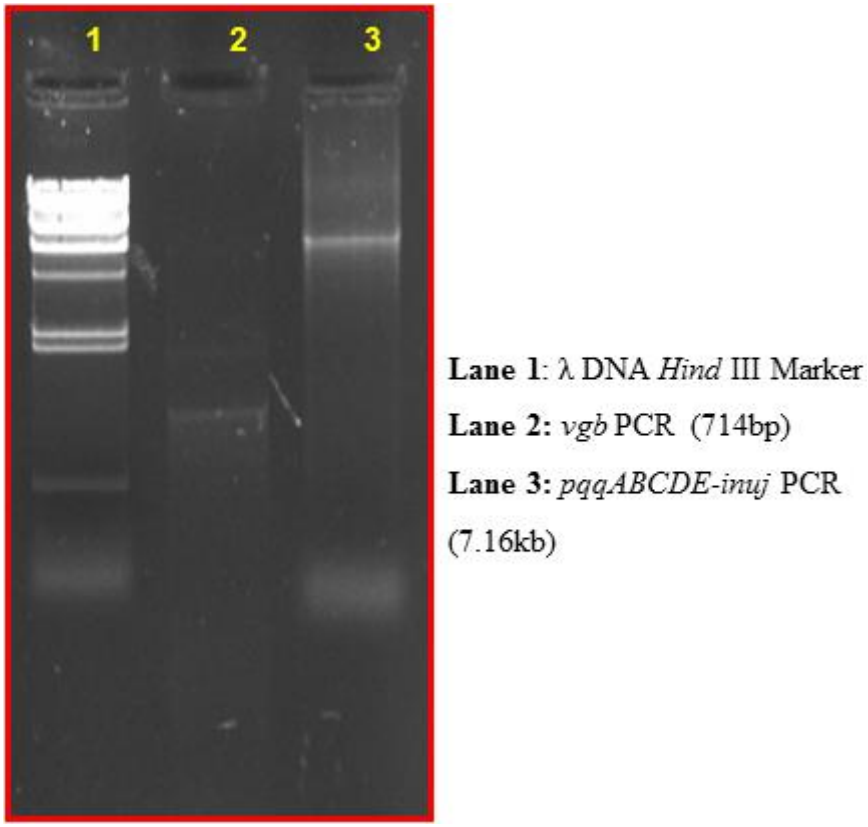
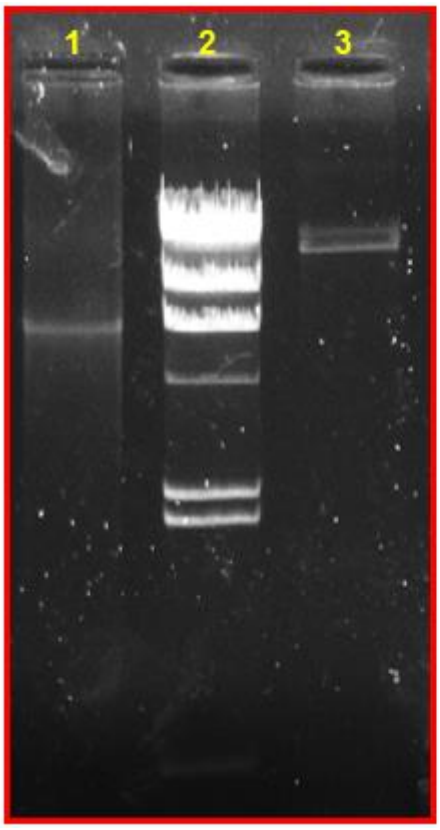


Fig. 4.14 Confirmation of recombinant pGRG36 *vgb-gfp-pqqABCDE-inuj* by gene specific PCR.

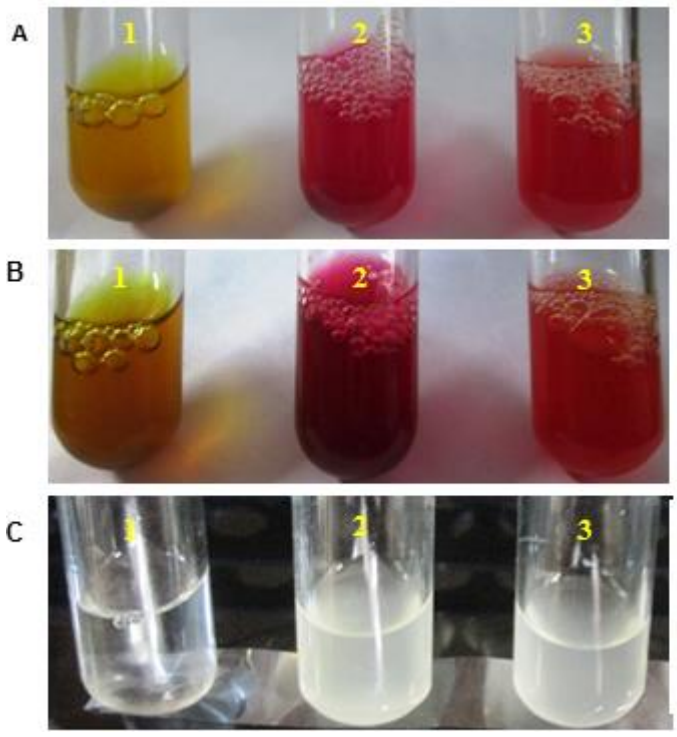


Lane 1: *pqqABCDE-inuj* PCR 7.16kb

Lane 2: λ DNA *Hind* III Marker

Lane 3: pGRG36 *vgb-gfp-pqqABCDE-inuj* digest with *Xho*I

Fig. 4.15 Restriction digestion of pGRG36 *vgb-gfp-pqqABCDE-inuJ*



Tris Buffered Media- Methyl Red
100mM-Tris; 100mM Glucose
1: EcN W/T
2: EcN-7
3: EcN-9

Tris Buffered Media- Methyl Red
100mM-Tris; 100mM Glucose
1: EcN W/T
2: EcN-8
3: EcN-10

M 9-Minmal Media (Sucrose)
1: EcN W/T
2: EcN-8
3: EcN-10

Fig. 4.16 Functionality of modified EcN harboring *pqqABCDE* and *pqqABCDE-inuJ* on Tris buffered and minimal M9 media containing sucrose as carbon source, respectively.

Both EcN-9 and 10 exhibited red colour formation in Tris buffered medium indicating gluconic acid production and functionality of *pqqABCDE* gene cluster **(Fig 4.16)**. EcN-10 grew on minimal medium containing sucrose reveling functionality of *inuJ* gene.

4.3.2 Effect of plasmid and genomic integrants of EcN on Body weight, fasting serum glucose and serum insulin levels

Body weight of sucrose control rat group and the rats receiving EcN-2 supplementation showed a marked increase in comparison to the control rats **(Fig. 4.17)**. However, rats fed with EcN-7 and 10, respectively, showed certain degree of reduction in their body weights although it was less in comparison to the rats receiving EcN-8. Rats administered with EcN-8 showed almost same body weight as that of control rat group. Interestingly, reduction in body weight was almost similar in rat groups supplemented with EcN-7 and EcN-10, respectively.

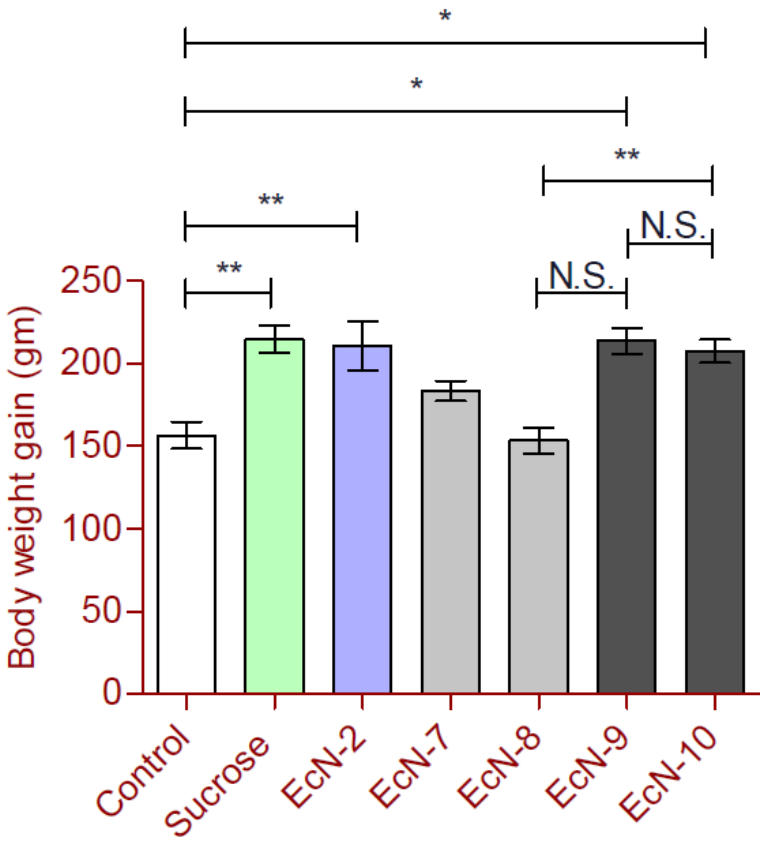


Fig. 4.17 Body weight gain of different rat groups over the period of the experiment. *P ≤ 0.05, **P ≤ 0.01.

Fasting blood glucose **(Fig. 4.18 A)** and serum insulin **(Fig. 4.18 B)** levels were monitored, as sucrose over-consumption is linked with insulin resistance. The blood glucose and insulin levels of rat groups administered with EcN-7, 8, 9 and 10, respectively, exhibited a significant decrease in comparison to sucrose control rat group. In this case as well, EcN-8 indicated maximum restoration, near to normal levels. EcN-8 supplementation showed similar trend as comprehended with body weight. EcN-2 did not exhibited any reduction in blood glucose and insulin levels suggesting these effects are attributed to PQQ, SCFA produced by fermentation of FOS and gluconic acid in gastrointestinal tract.

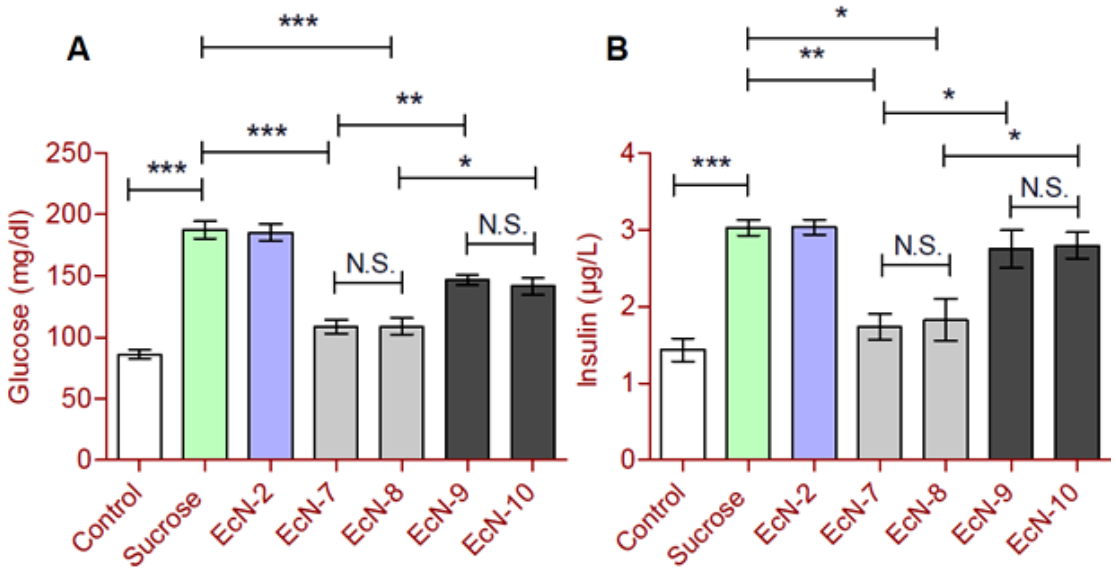


Fig. 4.18 (A) Fasting blood glucose and serum insulin levels. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, **P ≤ 0.01 and* P ≤ 0.001.**

4.3.3 Effect of plasmid and genomic integrants of EcN on serum and liver lipid profile

Consumption of sucrose is attributed to elevated serum triglyceride levels, a characteristic of metabolic syndrome. Serum triglyceride levels were found to be elevated in rat groups receiving only sucrose and EcN-2 supplementation, respectively **(Fig. 4.19)**. Significant reduction was witnessed in rat groups receiving EcN-7, 8, 9 and 10, respectively. Most pronounced reduction in serum triglyceride levels was found in the rats receiving EcN-8 dose. Here also, genomic integrants of *pqqABCDE* and *inuJ* showed effects close to as observed in EcN-7 harboring *pqqABCDE* gene cluster as plasmid clones. Cholesterol levels did not exhibited any significant difference in all the rat groups. However, serum HDL levels were found to

be significantly higher in EcN-7 and 8 administered groups with EcN-8 fed rat group showing maximum restoration.

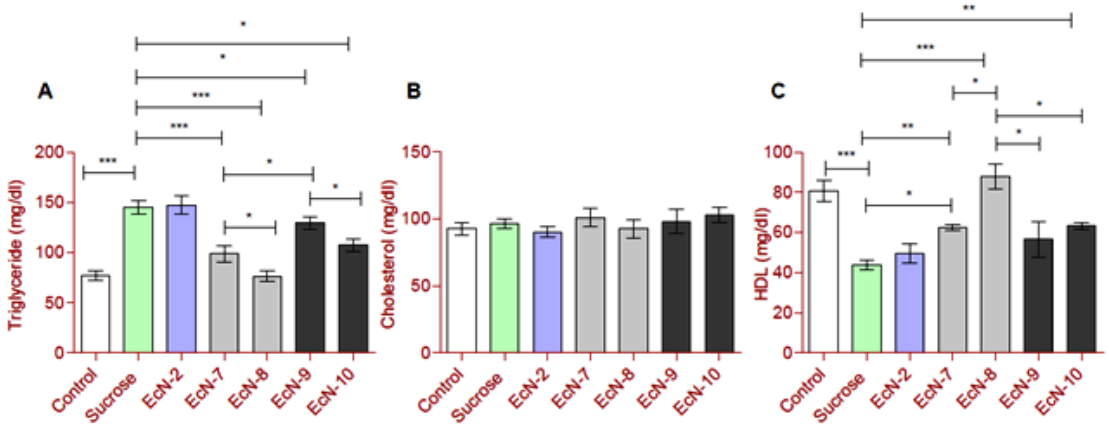


Fig. 4.19 Serum lipid profile: (A) Triglyceride, (B) Cholesterol and (C) HDL Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, **P ≤ 0.01 and *** P ≤ 0.001

To further investigate these effects, hepatic triglyceride levels were monitored **(Table. 4.4)**. Sucrose control rats showed maximum triglyceride accumulation which is in accordance with high serum triglyceride levels. Moreover, there was commitment increase in cholesterol content in these rats. EcN- 2 administration did not alter these parameters. However, supplementation with EcN-7, 8, 9 and 10 showed reduction in triglyceride and cholesterol levels with more pronounced effect in plasmid clones.

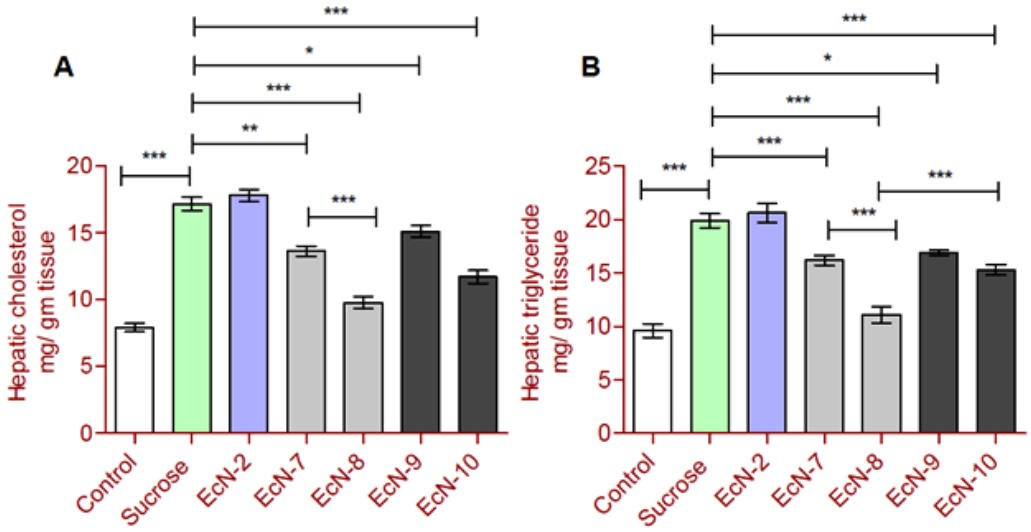


Table 4.4 Liver lipid profile of rats. Values are expressed as mean ± SEM (n=6 each group).

***P ≤ 0.05, **P ≤ 0.01 and *** P ≤ 0.001. Values are expressed as mg/g Tissue.**

4.3.4 Effect of plasmid and genomic integrants of EcN on serum and liver antioxidant status

Systemic oxidative stress is associated with sucrose consumption. As PQQ is also antioxidant molecule in addition to co-factor; antioxidant status was monitored in all groups. Activities of catalase and superoxide dismutase (SOD) were found to almost normal to control group in group administered with EcN-7 and 8 **(Fig. 20)**. Genomic integrants also exhibited significant increase in CAT and SOD activities, however, it was lower in comparison to plasmid harboring EcN. As Fructose is metabolized solely in liver antioxidant status was determined. Similar to that observed in the case of blood, hepatic lipid peroxidation also exhibited marked increase in sucrose control group **(Fig. 21)**. Administration of EcN-7, 8, 9 and 10 reduced ROS however maximum antioxidant effect was observed in case of plasmid based probiotics.

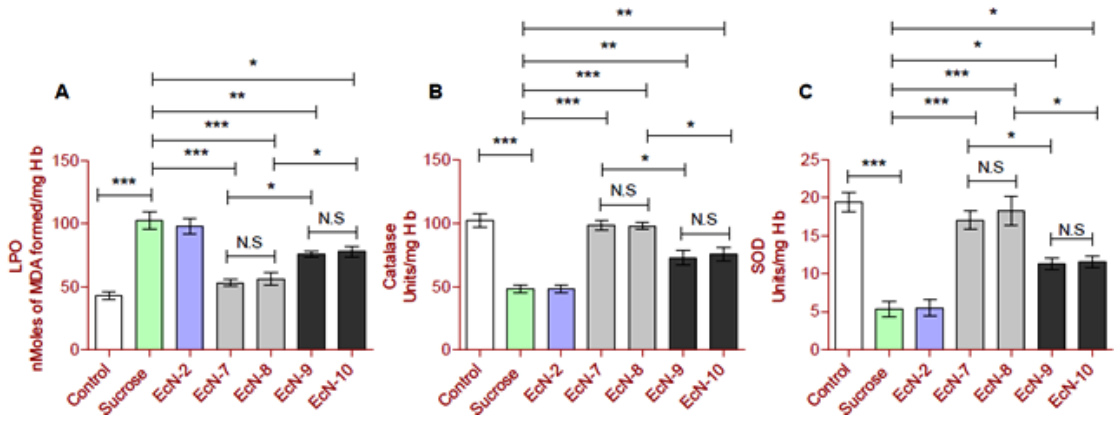


Fig. 4.20 Blood antioxidant status: (A) LPO, (B) Catalase and (C) SOD. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, **P ≤ 0.01 and *** P ≤ 0.001.

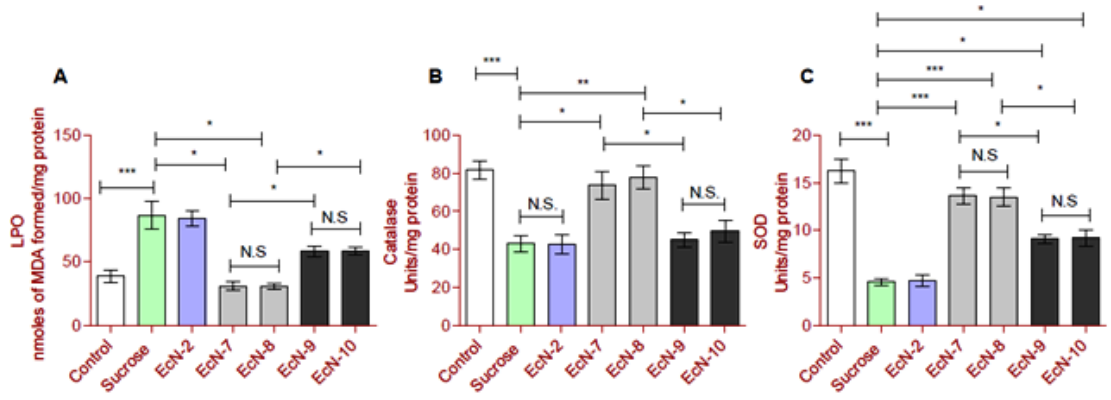


Fig. 4.21 Liver antioxidant status: (A) LPO, (B) Catalase and (C) SOD. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, **P ≤ 0.01 and *** P ≤ 0.001.

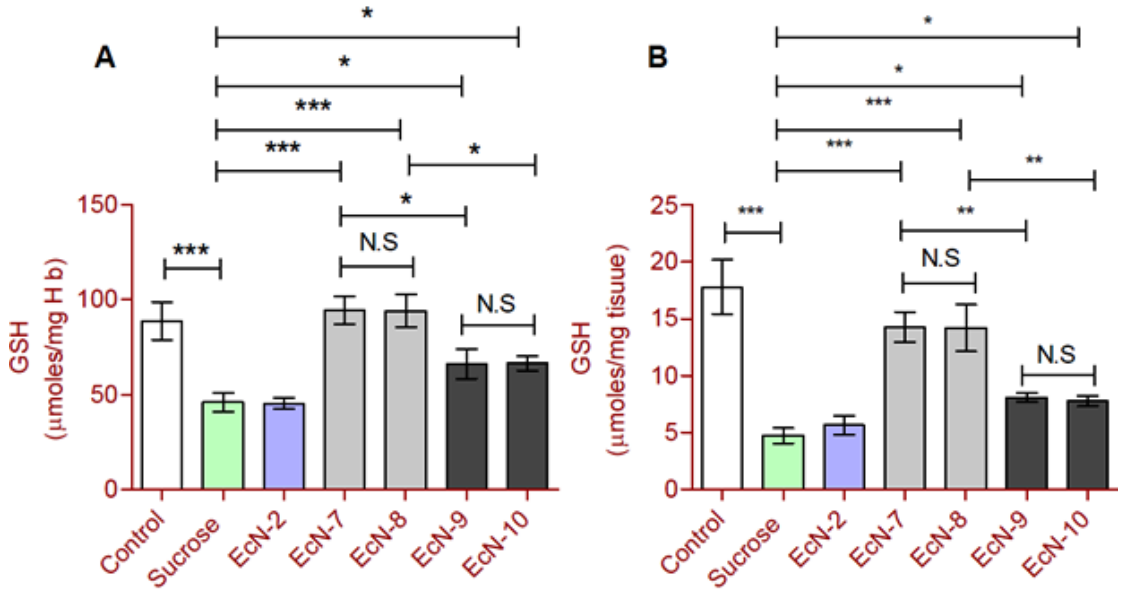


Fig. 4.22 (A) Blood and (B) liver GSH levels. Values are expressed as mean ± SEM (N=6 each group). *P ≤ 0.05, **P ≤ 0.01 and *** P ≤ 0.001.

Being an important marker for oxidative stress, reduced glutathione (GSH) levels were monitored in both blood and liver **(Fig. 4.22)**. Significant decrease in GSH levels were found in the sucrose control group. Similar results as observed in case of blood and liver CAT and SOD was also found in case of GSH. Here also, EcN-7, 8, 9 and 10 exhibited significant increase in GSH levels with maximum effect observed in case of EcN harboring *pqqABCDE* and *pqqABCDE-inuJ* plasmid.

4.3.5 SCFA profile

Colonic fermentation of gluconic acid produced from EcN glucose dehydrogenase and fructo-oligosaccharide formed by inulosucrase by *Lactobacillus* and *Bifidobacterium* species results in the production of SCFA. These SCFA are known to play important role in energy metabolism.

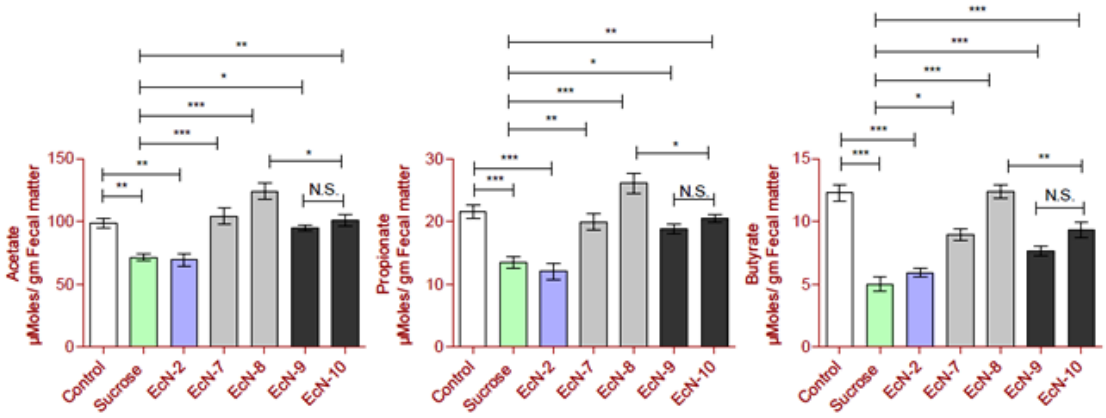


Fig. 4.23 Colonic SCFAs. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, **P ≤ 0.01 and *** P ≤ 0.001.

Quantification of SCFA from colonic content also reflects functionality of both *pqqABCDE-inuJ* genes. Rats administered with both genomic integrants of *pqqABCDE-inuJ* and plasmid based expression of *pqqABCDE-inuJ* resulted in increased SCFA content **(Fig 4.23)**. However, maximum SCFA production was observed in case of EcN-8 administered rats.

4.3.6 Fecal gluconic acid concentration

As mentioned previously, EcN glucose dehydrogenase results in gluconic acid production; we measured gluconic acid levels in colonic content of all the rat groups. Similar trend was observed as in case of SCFA profile. Gluconic acid quantification from colonic content also reinforces functionality of *pqqABCDE* gene cluster as observed *in-vitro* **(Fig. 4.24)**.

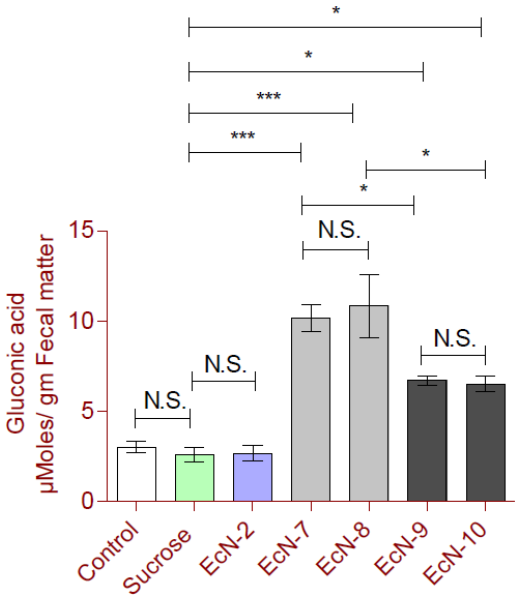


Fig. 4.24 Fecal gluconic acid Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, **P ≤ 0.01and ***P ≤ 0.001.

4.3.7 Fecal PQQ concentration

Fecal PQQ concentration was also monitored in all groups. Fecal PQQ concentration was found to be maximum in rats fed with EcN harboring *pqqABCDE* gene cluster in plasmid **(Fig. 4.25)**. However, genomic integrants also exhibited significant increase in fecal PQQ concentration albeit it was lower compared than plasmid constructs.

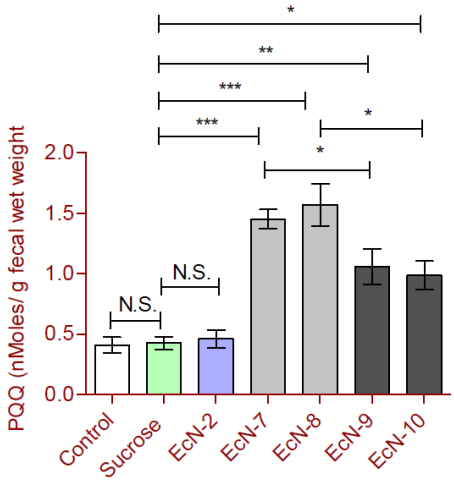


Fig. 4.25 Fecal PQQ quantification Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, **P ≤ 0.01 and *** P ≤ 0.001.

4.4 Discussion

Excessive intake of sucrose rich diets is attributed to increase risk associated with detrimental metabolic effects **(Tappy et al., 2010)**. In this study, rats fed with 20 % sucrose in drinking water daily for 65 days, exhibited perturbed metabolic status induced by sucrose consumption. In addition these rats displayed marked increase in the body weights. This observation is supported by evidences demonstrating consumption of sucrose rich diets can accelerate obesity, prime facie accompanied by the development of excess abdominal body fats **(Goran et al., 2013)**. However, groups administered with EcN-7 and 10 restricted body weight of these rats to some extent, with maximum effect in group receiving EcN-10. It is known that sucrose intake is associated with increased insulin resistance and increased fasting serum glucose levels. In context to fructose metabolism, which primarily occurs in the liver, hepatic inflammation, development of glycation products and hyperuricemic conditions is also associated with generation of oxidative stress **(Ohara et al., 1993; Lim et al., 2010)**. Rats on 20 % sucrose without probiotic treatment, exhibited a marked decrease in catalase and superoxide dismutase activity along with increased lipid peroxidation and reduced glutathione levels in liver and blood, where maximum sucrose induced oxidative stress has been proposed to be generated **(Lim et al., 2010, Tappy et al., 2010)**. EcN-7, 8, 9 and 10 secreting PQQ and PQQ-InuJ significantly restored these parameters with most pronounced effects observed in case of plasmid based EcN. This is attributed to fact that *pqqABCDE* and *pqqABCDE-inuJ* plasmid harboring EcN has multiple plasmids therefore physiological effect observed in case of plasmid is maximum. In contrast, genomic integrants of *pqqABCDE* and *pqqABCDE-inuJ* has only copy of *pqqABCDE-inuJ*. Improved antioxidant status is due to the antioxidant property of PQQ. Naturally occurring quinones have antioxidant property and they are used as drug to protect cells from oxidative stress *In-vivo*. Previously reported studies from literature support the fact that antioxidant property of PQQ is implicated in various beneficial effects including protection of neurological cells by suppressing peroxynitrile formation and scavenging of superoxide radical **(Misra et al., 2012)**.

Serum lipid profile was found to be elevated in the rats fed with sucrose rich water, in comparison to the control ones. Increased triglycerides levels were restored

significantly by administration of both plasmid and genomic integrants of *pqqABCDE* and *pqqABCDE-inuJ*. Here also plasmid based EcN showed significantly more restoration of serum lipid profile close to normal value with maximum effect in case of EcN-8. This observation could be attributed to synergistic effect of both *inuJ* and *pqqABCDE* towards lowering serum triglyceride level and increasing HDL levels. This finding is supported by experiments on hamster models, which demonstrated an elevated VLDL concentration and reduced HDL, after the administration of high sucrose diet **(Haidari et al., 2002)**. Moreover, synergistic effect of *pqqABCDE* and *inuJ* was also observed in context to the amount of short chain fatty acids in the fecal sample of the rats. Rats treated with EcN-7, 8, 9 and 10 exhibited elevated amount of SCFA in their feces. This increase was more prominent in the rats treated with Ecn-7 and 8 in comparison to EcN-9 and 10. Furthermore, this reflects the effective expression of inulosucrase resulting in formation of fructose oligosaccharides (prebiotic) which can be metabolized by colonic bacteria resulting in SCFAs as end product. Studies on animal models and human subjects have demonstrated that colonic fermentation of FOS results in SCFA production. Moreover, FOS acts as a soluble fiber and can be used to increase intestinal motility, transport and reduction of elevated plasma cholesterol **(Crittenden et al., 1996)**. In contrast to this, PQQ is a known co-factor for bacterial dehydrogenase and can aid to the synthesis of gluconic acid in *E. coli* **(Cleton-Janson et al., 1990)**.

Genomic integration of *inuJ-pqqABCDE* (7.16 kb) in the genome of EcN established a stable expression system, ruling out the possibility of plasmid loss. Reports support the fact that bacterial cells exhibit horizontal gene transfer via plasmid, phages or naked DNA. This could be matter of concern as plasmids can cross many species and genus barriers **(Thomas et al., 2005)**. Henceforth, allowing resistance to spread and persist in niches that are not necessarily subjected to antibiotics. Also, in the absence of antibiotics, hosts carrying multiple copies of resistance genes would be at a disadvantage due to the extra cost of producing more antibiotic resistance proteins. In addition to *pqq-inuJ*, *vgb-gfp* genes have also been integrated in the genome of EcN which resulted in improved colonization as mentioned in chapter 3. This is in accordance with published literature which has demonstrated that expression of *vgb* gene increases the effective intracellular oxygen concentration under micro-aerobic conditions, and improves growth of *E. coli* under

oxygen-limited conditions **(Khosla et al., 1988)**. Green fluorescent protein *(gfp)* confers was used as tag to identify our strain amongst gut microflora present in the intestine. Proposed mechanism of EcN producing PQQ and InuJ is represented in **Fig. 4.26 and 4.27.**

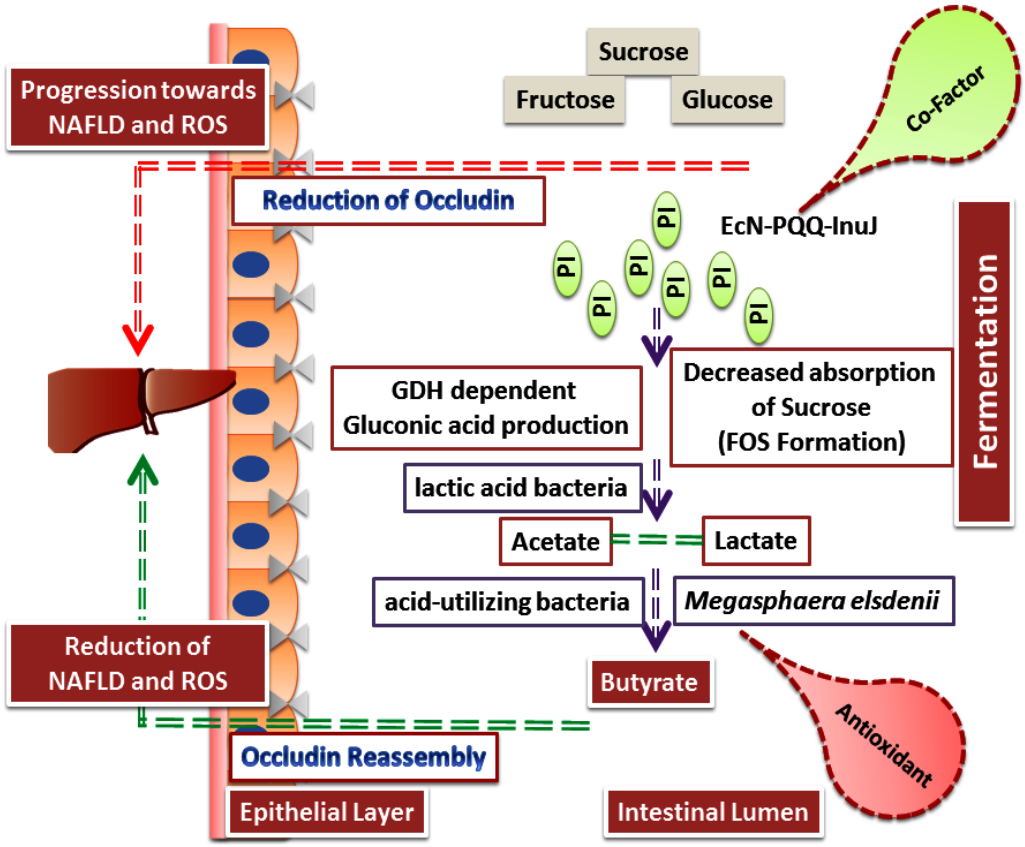


Fig. 4.26 Proposed mechanism of EcN producing PQQ and InuJ (A): Fructose component of sucrose is known to cause reduction of occluding. EcN is known to promote occluding assembly by its probiotic properties. In addition, modified EcN producing PQQ and InuJ can convert sucrose to Fructose oligosaccharide (FOS) in addition to production of gluconic acid by glucose dehydrogenase enzyme. Prebiotic (FOS and gluconic acid) so formed are metabolized by lactic acid bacteria in lower part of gastrointestinal tract resulting in production of acetate and lactate. Lactate so formed is metabolized by acid utilizing bacteria like *Megasphaera elsdenii* in end product such as butyrate and propionate. These SCFAs are key players in energy metabolism. PQQ apart from being co-factor for bacteria dehydrogenase is an excellent antioxidant molecule. Overall metabolic effect observed in the present study strongly suggests that these are synergetic of EcN, PQQ and InuJ.

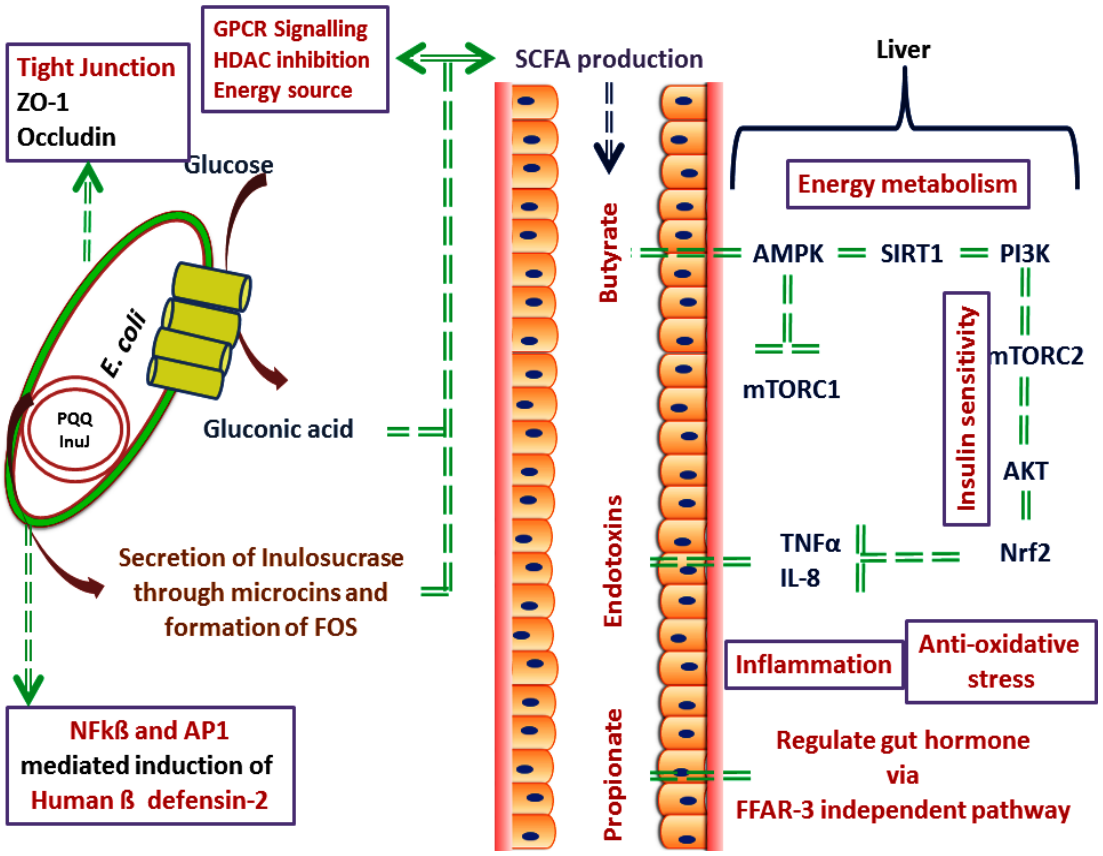


Fig. 4.27 Proposed mechanism of EcN producing PQQ and InuJ (B): SCFAs produced by the modified EcN can modulate energy metabolism, insulin sensitivity, inflammation, anti-oxidative stress and inflammation by different pathways as represented in the figure. EcN alone can induce Human ß defensing-2 via NFk ß and AP1.

Based on these findings it can be concluded that both genomic integrant and plasmid based expression systems were able to manage sucrose induced metabolic effect. However, the EcN-9 and 10 (genomic integrant) were less effective in comparison to plasmid based counterpart EcN-7 and 8. This finding is supported by the fact that genomic integrants exists as a single copy whereas plasmid based expression exists as multiple copies depending upon the copy number of the vector. Based on these observations, implementation of a strong promoter in genomic integrant is required for achieving expression level equivalent to that of plasmid based expression systems. In addition to these, the contribution from the short chain fatty acids behind maintaining metabolic homeostasis needs to be investigated.