Chapter 5

Effect of PQQ producing plasmid transformants and genomic integrants of probiotic *Escherichia coli* Nissle 1917 on colonic SCFA levels in starch fed rats

5.1 Introduction

Mammalian gastrointestinal tract is inhabited by enormous amount of bacteria with largest number of bacterial population is found in large intestine (10^{11} per gram fecal matter) (Tlaskalova-Hogenova et al., 2011). Large intestine is majorly anaerobic and bacterial population residing there are largely involved in fermentation. Many dietary components including non-digestible carbohydrates and other prebiotics are metabolized by colonic bacteria which produce several bioactive compounds such as SCFAs (Butyrate, propionate and acetate), conjugated linoleic acid (CLA) and equol (Davis & Miller, 2009). These bioactive compounds have several beneficial roles in maintaining optimal host health.

SCFAs are the major group of metabolites produced by gut microbe (Besten et al., 2013). They are considered as necessary waste product to the gut microbial community, since it is required to balance redox equivalents produced in anaerobic environment of the gut (Hoek and Merks, 2012). SCFAs are saturated aliphatic organic acids containing one to six carbons, out of which acetate (C2), propionate (C3) and butyrate (C4) represents more than 95 % of total SCFAs present in the colon (Besten et al., 2013). Acetate is found comparatively in higher concentration than propionate and butyrate. Approximately 95 % of SCFAs produced in the gut are rapidly absorbed and the rest is excreted through feces. SCFAs are transported across intestinal epithelia via diffusion of lipid soluble form (SCFA/HCO_3^- exchange) and actively through SCFA transporters (Hamer et al., 2008). Butyrate is major energy source for colonocytes, however, SCFAs can also be found in hepatic tissues, portal and systemic blood (Cummings et al., 1987; Murase and Nagata, 1995). Majority of absorbed butyrate is metabolized by colonocytes and remaining goes to liver for further extraction via portal vein.

In last few decades, extensive research on SCFAs has led to the notion that they are very important for host health and play a key role in prevention and treatment of metabolic disorders, bowel disorders (i.e. Inflammatory bowel

disease) and colon cancer (Hamer et al., 2008; Gao et al., 2009; Hu et al., 2010; Tang et al., 2011; Besten et al., 2013; Tan et al., 2014). SCFAs regulates fatty acid metabolism by activating their oxidation and inhibiting de novo synthesis and lipolysis, leading to reduced plasma free fatty acid and decreased body weight (den Besten et al., 2013; Puertollano et al., 2014). Moreover, protective effect of butyrate against H_2O_2 induced oxidative DNA damage has also been reported in some of the studies, possible through affecting enzymatic and non-enzymatic antioxidant systems (Abrahamse et al., 1999; Rosignoli et a., 2001). Butyrate, at low concentration, also regulates intestinal permeability by modulating intestinal tight junction gene expression in mammalian cell culture models (Mariadason et al., 1997; Peng et al., 2007).

E. coli is known to express membrane bound Glucose dehydrogenase (GDH) enzyme, which catalyzes the conversion of glucose to gluconic acid using external PQQ as co-factor. Gluconic acid is then used up by some colonic bacteria for energy generation (Tsukahara et al., 2002). Interestingly, *E. coli* does not possess genes for production of PQQ, hence GDH is in apo-form and depends upon PQQ present in the environment. However, in absence of PQQ in the medium, *E. coli* does not exhibit any growth defects.

Plasmid based expression systems are unstable because of huge possibility of plasmid loss during time. Moreover, presence of plasmid exerts metabolic load on the cell hindering its growth and metabolic status (Sharma et al., 2005). Markerless integration of genes can be achieved by transposon mediated gene integration (McKenzie *et al*., 2006). In the present study *pqqABCDE* gene cluster from *G. suboxydans* 621H was sub-cloned under constitutive *tac* promoter followed by genomic integration in *E. coli* Nissle 1917 genome. Genomic integration results in single copy of the genes in the genome, hence cloning *pqqABCDE* genes under strong constitutive *tac* promoter was attempted to enhance PQQ production. This study we transformed probiotic EcN in symbiotic EcN capable of producing prebiotic gluconic acid which is further metabolized by gut microbiota to produce beneficial SCFAs.

5.2 Methods and materials

5.2.1 Bacterial strains and culture conditions

Different bacterial strains and plasmid constructs used in this study are summarized in **Table 5.1**.

Table 5.1plasmids and bacterial strains used in the present study

Plasmids/ Strains	Characteristics	References
pMAL_P2	Periplasmic expression vector	Zwick 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 work
6A-*PQQ*-2	pMAL_P2 Δ Lac^{Iq} with *pqqABCDE* (Bgl II-Xba I sites)	This work
PGRG_36-VGP	pGRG_36 integration vector harboring *vgb*, *gfp* and *pqqABCDE* genes	This work
ECN	Probiotic *E. coli* Nissle 1917	Sonnenborn and Schulze et al., 2009
ECN-2	EcN strain with genomic integration of *vgb*and *gfp*genes.	Chapter-2
ECN-6	EcN-2 harboring 6a (pMAL_P2 Δ Lac^{Iq}) plasmid	This study
ECN-7	EcN-2 harboring 6a-*pqq*-2 plasmid	This study
ECN-9	EcN strain with genomic integration of *vgb*, *gfp* and *pqqABCDE* genes (using pGRG36-vgp plasmid)	This study

5.2.2 Construction and characterization of EcN producing PQQ

G. suboxydans 3.7 Kb *pqqABCDE* genes, along with upstream and downstream open reading frames, were amplified using PQQ-F-Bgl II (GAAGATCTTCGCGGATGTTCAGGTGTTCGC) and PQQ-R-Xba I (ATCGTCTA GAAGAAGATGGCCTCTCCTGGG) primers and cloned in pTZ57 R/T cloning vector (Thermo Scientific) generating plasmid pTPQ-2. Restriction enzyme (RE) digestion of pTPQ-2 plasmid with Xba I released 3.7 Kb *pqqABCDE* containing fragment, which was further ligated with vector backbone of pMal_P2 ΔLac^{Iq} (predigested with Xba I) generating 6a-pqq-1 plasmid. 6a-pqq-1 plasmid express

pqqABCDE operon only from native *pqqABCDE* promoter. RE digestion of pTPQ-2 plasmid with Bgl II and Xba I released 3.7 Kb *pqqABCDE* containing fragment, which was further ligated with vector backbone of pMal_P2 ΔLac^{Iq} (predigested with Bgl II and Xba I) generating 6a-pqq-2 plasmid. 6a-pqq-2 plasmid express *pqqABCDE* operon from two promoters, first native *pqqABCDE* promoter and second from vector strong *Tac* promoter. *pqqABCDE* operon from 6a-pqq-2 was amplified using cTac-F (5' TTGACAATTAAT CATCGGCTCGTATAATGGATCGAATTGTGAG 3') and Reverse (5' CGCTTCT GCGTTCTGATTTAATCTGTATCAGG 3'), and were cloned (blunt end ligation) in to linearized pGRG36-*vgb-gfp* (digested with Xho I and end filled using Klenow fragment) generating pGRG36-vgp plasmid.EcN was transformed with 6a-pqq-2 and pGRG36-vgp (for genomic integration) plasmids generating EcN-7 and EcN-9 strains. Genomic integration was performed using temperature sensitive Tn7 mediated integration system (McKenzie *et al*., 2006). *vgb*gene was amplified using Vgb-F GCGCGGAATTCATGTTAGACCAGCAAACCATTA ACATCATC and Vgb-R GCGCGCTCGAGTTATTCAACCGCTTGAGCGT ACAAATCTG for confirmation of genomic integrants.

5.2.3 Animal experiments

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). Rats were allocated randomly in different groups and received standard defined starch rich diet with full access to drinking water **(Figure 5.1 &Table 5.2)**.

Table 5.2 Diet composition used for the study (Cohen et al., 1972)

Composition of standard defined starch rich diet		**Vitamin mixture: Composition of vitamin mixture per kg**		**Salt mixture: - Composition of salt mixture per kg**	
Ingredients	*Content (%)*	*Ingredients*	*Content*	*Ingredients*	*Content*
Corn starch	64g	Vitamin A concentrate	2500 I.U	$MgSO_4$ $7H_2O$	30.5 g
Casein powder	20g	Vitamin D3 Cholecalciferol	200 I.U	NaCl	65.2 g
Wheat bran	9.7g	Thiamine hydrochloride	0.5 mg	KCl	105.7 g
Ground nut oil	5ml	Riboflavin	0.5 mg	KH_2PO_4	200.2 g
Salt mixture	3.5g	Pyridoxin	0.5 mg	$MgCO_3$	3.65 g
Methionine	0.7g	Sodium pantothenate	1.5 mg	Mg (OH) $_2$. 3H2O	38.8 g
Vitamin mixture	0.2g	Nicotinamide	5 mg	$FeC_6H5O_7.5H_2O$	40.0 g
Chlorine chloride	0.2g	Ascorbic acid	25 mg.	$CaCO_3$	512.4 g
				KI	0.8 g
				Na F	0.9 g

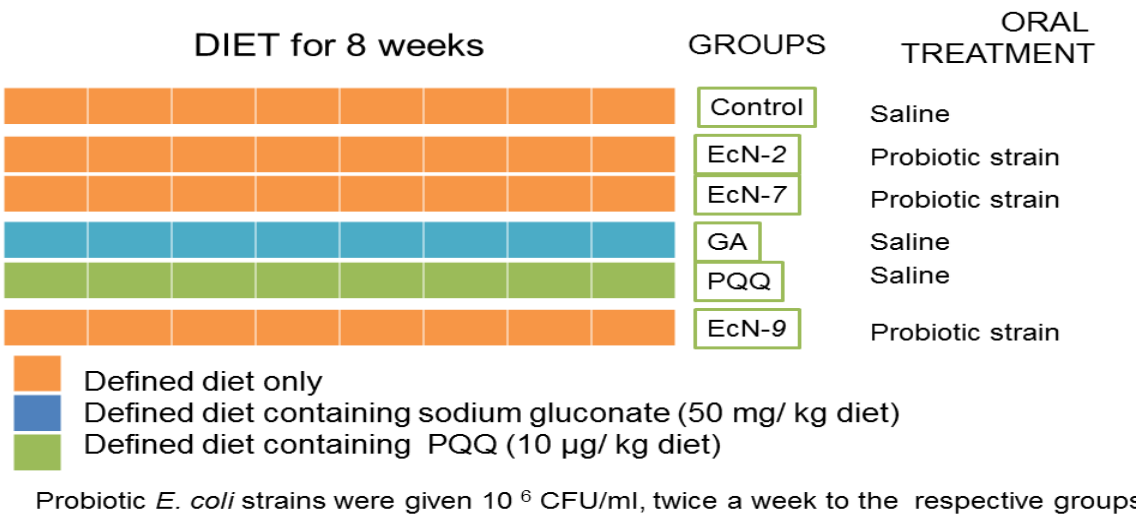

Figure 5.1 Strategy for animal experimentation. Rats were randomly divided in 6 groups (6 animals each group).

5.2.4 Sample preparation and Biochemical estimations

PQQ was extracted from *E. coli* Nissle harboring *pqqABCDE* gene cluster by using methods described by Suzuki *et al*. (1990) with slight modification as described in previous chapters. Fecal sample stored at -80^{0}C were thawed, re-suspended in sterile PBS and centrifuged. Supernatant was taken for PQQ estimation. Stored Feces were also used for gluconic acid SCFAs estimations. Concentration of gluconic acid and SCFAs were analyzed by using HPLC method.

For gluconic acid estimation, sample preparation for HPLC was done by method described by Roukas, (2000). Briefly, cells grown in M 9 minimal media containing glucose as a carbon source for overnight at 37^{0}C. Supernatant was collected by centrifugation at 10000 g for 30 minutes at 4^{0}C. Further supernatant was filtered with 0.2 micron filter. It was stored at -80 0C till use. Fecal samples stored at -80^{0}C were thawed and homogenized in ice cold water (HPLC grade). Then homogenate was centrifuged at 10000 g for 30 minutes at 4^{0}C. Supernatant filtered with 0.2 micron filter was used for injection. Standard graph using defined concentration of Gluconic acid was prepared for reference. HPLC conditions were – Column: C 18column; System: Waters® HPLC system; Flow rate: 1 ml/ min; Column temperature: Room temperature; Retention time: approximately 3 minutes; Mobile phase: 5 mM H_2SO_4 (pH= 2.5; adjusted with Na OH); Detector: UV (210 nm).

For SCFAs estimation fecal samples stored at -80^{0}C were thawed and homogenized in HPLC grade water. Homogenate was centrifuged at 10000 g for 30 minutes at 4^{0}C. Supernatant filtered with 0.2 micron filter was used for injection. Standard graphs using defined concentrations of SCFAs were prepared for reference. HPLC conditions were - Column: C 18 column. (Broad pH range); System: Waters® HPLC system; Flow rate: 0.6 ml/ min; Column temperature: Room temperature; Mobile phase: 0.01 N H_2SO_4 (pH= 2.5; adjusted with Na OH); Detector: UV (210 nm).

5.2.5 Statistical analysis

Statistical analysis was done using Graph pad PRISM version 3.0 and one-way ANOVA. (p value ≤ 0.05, considered as significant).

5.3 Results

5.3.1 Generation of high PQQ producing EcN strain and genomic integration of pqqABCDE gene cluster in EcN.

In order to confirm the recombinant, plasmid was isolated from EcN transformant and subjected to restriction enzyme digestion. Bands were visualized after gel electrophoresis **(Figure 5.2, 5.3 and 5.4)**.

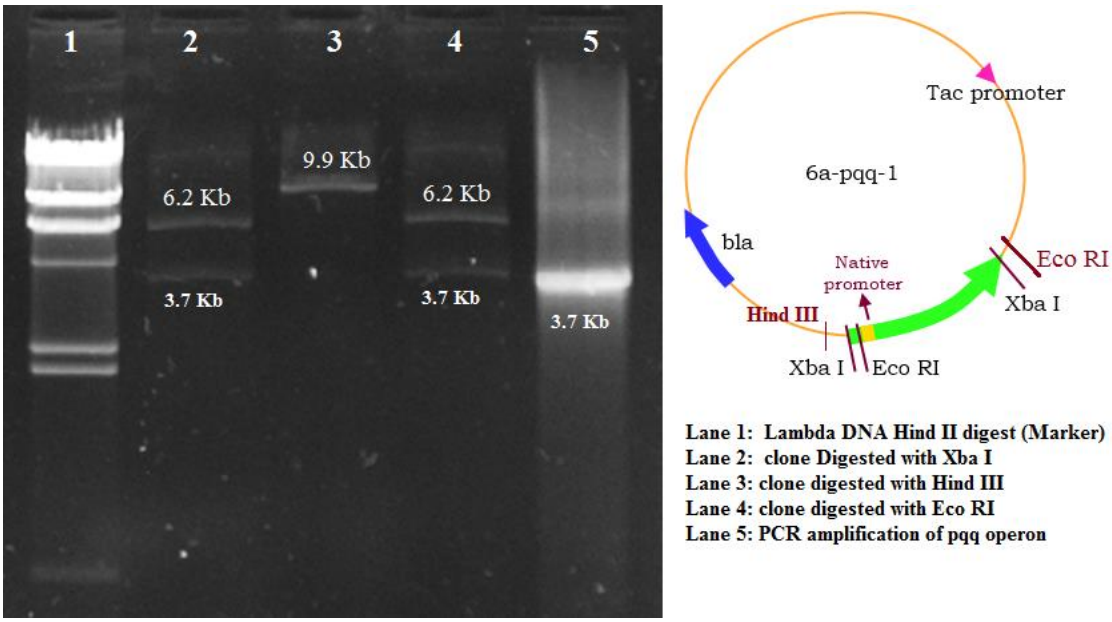

Figure 5.2 Cloning and confirmation of 6a-*pqq*-1 plasmid. Lane-2 shows two bands; a 3.7 kb of *pqq* gene band and a 6.2 kb of vector backbone. Lane-3 shows linearized plasmid of 9.9 kb containing *pqq* and 6a vector. The lane-4 represents two bands; a 3.7 kb of *pqq* gene and 6.2 kb of vector backbone which confirms that *pqq* gene inserted in opposite orientation of c*tac* promoter. Lane-5 shows PCR product of *pqq* gene which is similar in size with insert release of lane-2 and lane-4.

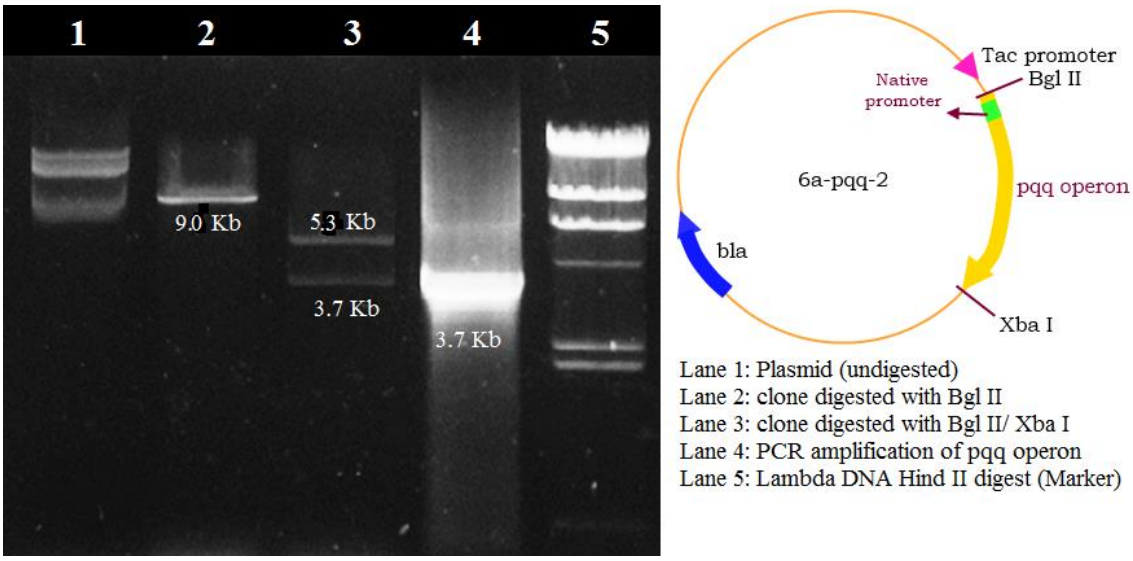

Figure 5.3 Cloning and confirmation of 6a-*pqq*-2 plasmid. Lane-2 shows the linearized plasmid consist of *pqq* and 6a vector digested with Bgl II which of 9 kb. Lane-3 shows two bands; a 3.7 kb band of *pqq* gene and a 5.3 kb of vector backbone of 6a vector. Lane-4 shows PCR product of *pqq* which of 3.7 kb and similar with insert release of lane-3.

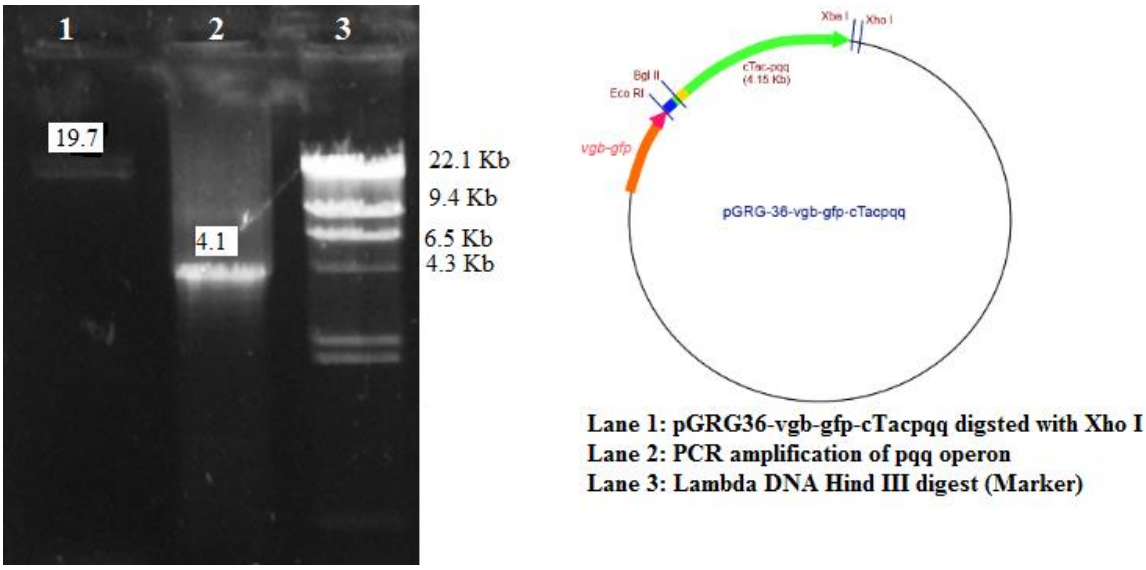

Figure 5.4 Cloning and confirmation of pGRG-36-*vgb-gfp*-c*Tacpqq* plasmid. Lane-1 shows linearized plasmid containing vector and *pqq* insert which of 19.7 kb. Lane-2 shows PCR product of *pqq* gene which of 4.1 kb.

EcN was transformed with pGRG-36-*vgb-gfp*-c*Tac-pqq*construct followed by genomic integration. Integrants were confirmed for the presence of GFP by visualizing under UV-transilluminator. Green fluorescent colonies showed the presence of GFP protein. Presence of *vgb* and *pqqABCDE* genes in genomic were confirmed by PCR using specific primers **(Figure 5.5)**.

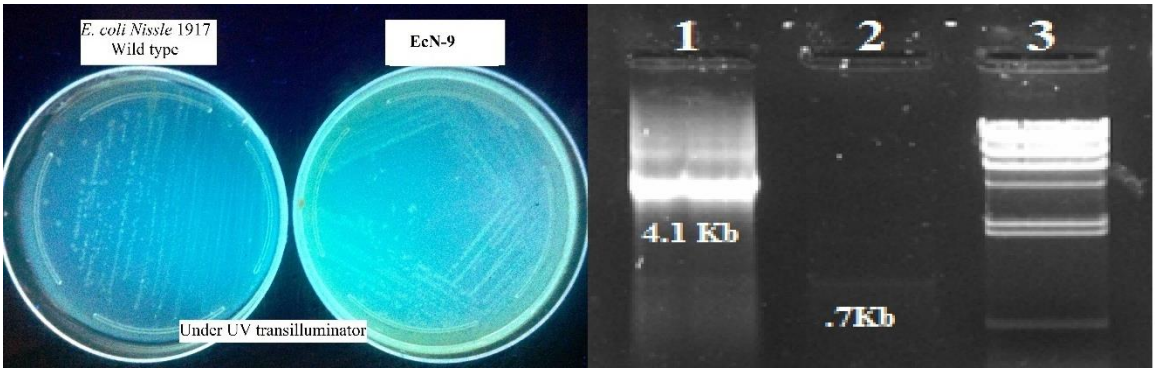

Figure 5.5 Confirmation of integration *vgb, gfp* and *pqqABCDE* genes in EcN genome. Gfp was confirmed by fluorescent colonies under UV-transilluminator and *vgb* and *pqqABCDE* genes were confirmed by PCR using specific primers. Lane 1- cTac-*pqqABCDE* PCR; Lane 2- *vgb* PCR and Lane 3- Lambda DNA Hind III digest.

After confirmation, recombinant EcN strains were grown in M9 minimal medium containing 100 mM glucose for 24 h. EcN-7 strain (carrying 6a-pqq-2) plasmid produces significant higher amount of PQQ in the medium as compared to EcN-7a (harboring 6a-pqq-1 plasmid) **(Table 5.2)**. Henceforth, EcN-7 was chosen for the further studies. EcN-2 and EcN-6 strains lacking *pqqABCDE* genes, does not produce PQQ.

Table 5.3 PQQ production in vitro by different EcN strains.

Strains	**EcN-2**	**EcN-7a**	**EcN-7**	**EcN-9**
PQQ (µg/ ml culture)	ND	2.26 ± 0.6	2.58 ± 0.14$^{@@@}$	1.12 ± 0.12 ***

*All values are expressed as mean ±SD of 3 independent experiments. Cells were grown in M9 minimal media containing 100 mM glucose for 24 hours. Supernatant was used for estimation of PQQ.$^{@@@}$p≤0.001 compared to EcN-7a,***p≤0.001 compared to EcN-7. ND means not detected.*

5.3.2 PQQ producing EcN-7 and EcN-9 secretes gluconic acid *in vitro* and *in vivo*

EcN-7 (harboring 6a-pqq-2) and EcN-9 (genomic integrants) also produced gluconic acid in vitro when grown in M9 minimal medium containing 100 mM glucose for 48 h. As expected, EcN-7 strain produced significantly higher amount of gluconic acid as compared to EcN-9 **(Figure 5.6 A)**. Moreover, when these strains are administered to rats by oral gavage, significantly higher concentration of gluconic acid was found in feces of these rats as compared to controls. Rats treated with EcN-7 had more gluconic acid in feces as compared to EcN-9 **(Figure 5.6 B & C)**.

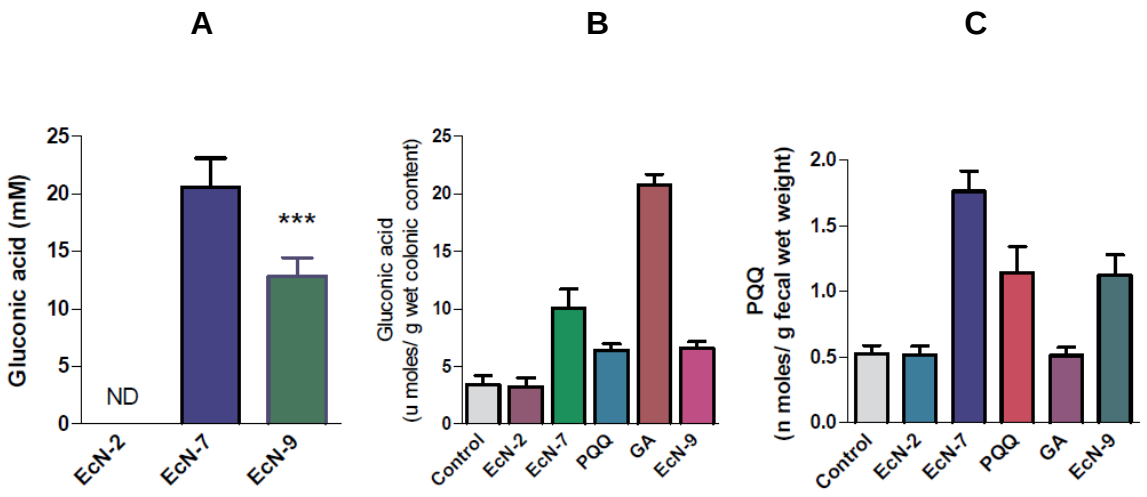

Figure 5.6 PQQ producing EcN strains secreting gluconic acid *in vitro* (A), *in vivo* (B) and PQQ in vivo (C). All values are represented as mean ± SD. 3 independent experiments (A). 6 rats per group (B and C).

5.3.3 Effect of EcN-7 feeding, dietary gluconic acid and PQQ on colonic SCFAs profile

SCFAs concentration was checked to demonstrate the prebiotic potential of EcN transformant **(Figure 5.7)**. Basal level of butyrate and propionate wasfound in control and EcN-2 treated groups, while significant increase was found in groups fed with EcN-7 and EcN-9 strains and sodium gluconate. Groups fed with EcN-9 and extracted PQQ showed significant lesser level of butyrate and propionate as compared with EcN-7 fed group. There was significant increase in acetate level in groups fed with EcN-7 (plasmid based expression) and Gluconic acid. However, no significant increase was seen in acetate concentration in other groups.

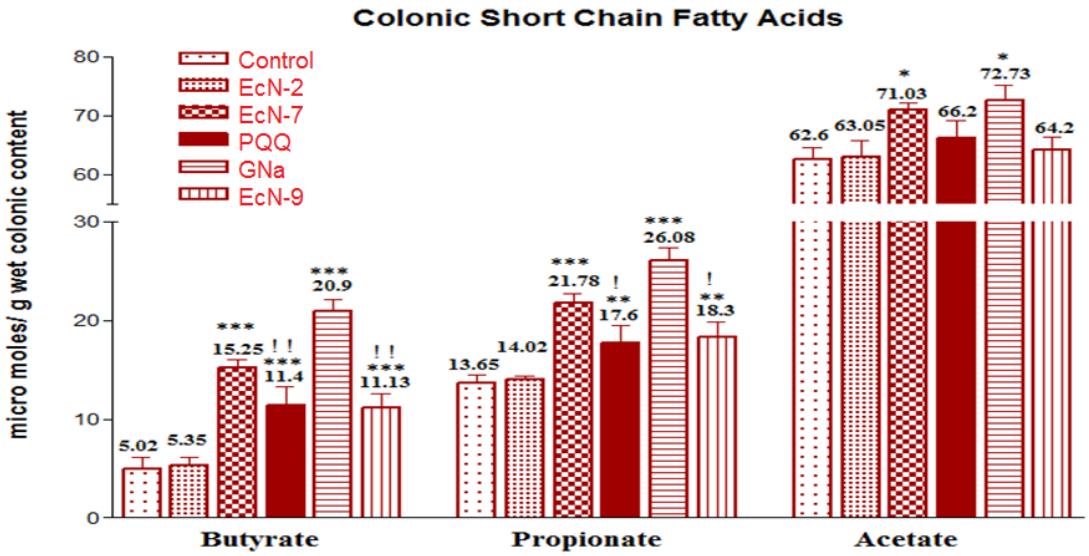

Figure 5.7 Colonic SCFAs profile in starch fed rats co-treated with recombinant EcN strains producing PQQ. Values are represented as Mean ± SD. (6 rats each group).*p< 0.05, *p< 0.01 and ***p< 0.001 compared to Control. !p< 0.05 and !! p< 0.01 compared to EcN-7.

5.4 Discussion

PQQ producing operon *pqqABCDE* from *G. suboxydans* 621H has been successfully cloned and expressed in *E. coli* strains (Yang et al., 2010). The present study demonstrates PQQ and gluconic acid production by EcN harboring *pqqABCDE* gene cluster from *G. suboxydans* 621H. *In vitro* experiments suggest that significantly higher but marginal increase in PQQ production (2.58 µg/ml) could be achieved by cloning *pqqABCDE* operon under constitutive *tac* promoter along with its native promoter in probiotic *E. coli* Nissle 1917 (EcN-7).

In presence of PQQ, glucose dehydrogenase becomes active and hence, PQQ producing strains produced gluconic acid *in vitro* and *in vivo*. This is consistence with the other reports which shown that *E. coli* able to produce gluconic acid if cofactor PQQ is available for its apo-GDH (Cleton et al., 1990; Adamowicz et al., 1991; Biville et al., 1991).

However, when this operon is integrated in the genome of *E. coli* Nissle, PQQ production and gluconic acid secretion is lowered by approximately 60 % and 50 %, respectively, which could be attributed to single copy of *pqq* gene present in genomic integrants.

Rats fed with EcN harboring *pqqABCDE* genes (plasmid/genome) and extracted PQQ showed significant increase in fecal PQQ and gluconic acid levels. However, group fed with extracted PQQ daily in diet showed lesser level of gluconic acid and SCFAs as compared to EcN-7 (plasmid based expression of *pqqABCDE*). This could be because of less availability of PQQ for gluconic acid production in these rats, since; major portion PQQ might get absorbed and lesser is left available to commensal *E. coli* present in the colon, which can produce gluconic acid through active GDH. Most importantly, PQQ secreting recombinant EcN strains are capable of increasing SCFAs concentration (Mainly butyrate and acetate) in starch fed rats. These results are consistent with previous studies which show that higher SCFAs concentration could be achieved by supplementation of gluconic acid (Tsukahara and Ushida et al., 2002; Biagi et al., 2006).It is important to note that genome based expression of PQQ (EcN-9) was

found to be less effective as compared to plasmid based (EcN7). However, organic acids estimated in colonic content were found still significantly higher in these rats as compared to control. To our knowledge, this is the first report demonstrating the prebiotic potential of gluconic acid secreted by EcN harboring *pqqABCDE* strains in healthy rats with increased SCFAs production in colon. The increase in SCFAs production by gluconic acid has previously been studied in human trials and colon cancer rat models (Kanauchi et al., 1999; Biagi et al., 2006).

Previous studies (Chapter 2 & 4; Pandey et al., 2014) from the lab showed increase in colonic SCFAs levels upon intragastric ingestion of PQQ producing probiotic *E. coli* strains (Nissle 1917 & CFR 16) in rats. The present study explains that the increase in colonic SCFAs by PQQ producing probiotic *E. coli* strains could also be mediated by generation of gluconic acid in the colon as intermediate substrate. Gluconic acid is selectively fermented by *Bifidobacteria* and *Lactobacilli* species (Asano et al., 1994). Feeding of sodium gluconate to piglets increases fecal content of gluconic acid and increase the proportion of *Bifidobacteria* (Tsukahara et al., 2002). *Bifidobacteria* and *Lactobacilli* produces acetate and lactate from gluconic acid which is utilized by *M. elsdenii* for butyric acid production. Feeding gluconic acid to animals was shown to increase levels of SCFAs (Acetate, propionate and Butyrate) in piglets and increasing concentration of gluconic acid resulted in linear increase of colonic SCFAs in these animals (Kameue et al., 2004; Biagi et al., 2006; Poeikhampha et al., 2011).

This proof of concept study suggest that EcN strains producing PQQ in the gut are capable of efficiently enhancing butyrate and propionate levels in colon, majorly by production of gluconic acid, and could be exploited in further studies as treatment options for various physiological disorders which are modulated by SCFAs. SCFAs have been implicated to reduce the risk of metabolic syndrome, many hereditary disorders, immunological disorder and colon cancers (Maslowski et al., 2009; Binder et al., 2010; Canani et al., 2011;

Vinolo et al., 2011; Canani et al., 2012). The present strategy of combining probiotic, prebiotic and PQQ has multifactorial benefits. It integrates (i) probiotic effects of (*E. coli* Nissle 1917), (ii) prebiotic activity of gluconic acid accompanied by increase SCFAs production, (iii) antioxidant potential & mitochondrial biogenesis function of PQQ, and (iv) Anti-lipidemic effects of PQQ and SCFAs. It would be interesting to develop EcN integrants secreting PQQ equivalent to that of plasmid transformants to achieve optimal benefits.

In conclusion, we showed that PQQ given in diet can be converted by commensal gut bacteria by active GDH to gluconic acid. Probiotic secreting PQQ acts as more efficient gluconic acid generator in the intestine as compared to PQQ given in the diet. The probiotic bacteria acts as symbiotic (probiotic secreting prebiotic). The gluconic acid generated can be metabolized by resident bacteria and as a result enhanced SCFAs are seen in the colonic content of the treated mice. It would be interesting to develop more efficient genomic integrants of *pqqABCDE* gene cluster capable of producing PQQ equivalent to plasmid based constructs.