Chapter 4:

Evaluating the efficacy of probiotic *E. coli* Nissle 1917 strain containing citrate synthase and *pqq* operon in amelioration of heavy metal mercury induced metabolic disorders in rat model.

4. Introduction

Mercury (Hg), an environmental heavy metal pollutant is considered toxic to the central nervous system, renal, dermatologic, cardiovascular, gastrointestinal, reproductive and immune system (Zalups *et al*., 2000; Goyer *et al.*, 2001; Virtanen *et al*., 2007; Holmes *et al*., 2009). Kidneys, liver and brain are the most affected organs by Hg exposure (Magos and Clarkson, 2006; Othman *et al*., 2014; Boroushaki *et al*., 2015). Oxidative stress is partly responsible for heavy metal pathogenesis. Hg is known to impart toxicity by molecular interactions with sulfhydryl groups, carboxyl and phosphoryl groups which are abundantly present in proteins and polypeptides (McGoldrick *et al.*, 2003; Geier *et al*., 2007; Rooney, 2007).The long term effective treatment of metal toxicity can be strengthen by either reducing the possibility of metal interacting with biomolecules or by enhancing the cells antioxidant defenses through endogenous supplementation of antioxidant molecules. Heavy metals impair prooxidant/antioxidant ratios that can be rebalanced by using antioxidants like vitamin-C, carotenoids, selenium, vitamin-E (Patra *et al*., 2011). Pyrroloquinoline quinone (PQQ) is more potent than these antioxidants as it has redox catalytic cycles of 20,000 (Rucker *et al*., 2009). Additionally, PQQ is known to act as growth factor, enhance reproductive capabilities, maintain mitochondrial, neuronal function, reduce inflammation and liver fibrosis (Debray *et al*., 2008; Rucker *et al*., 2009; Harris *et al*., 2013,Yang *et al*., 2014; Jia *et al*., 2015).

To overcome metal toxicity, Chelation therapy is extensively gaining approval. It works by balancing concentrations of essential metal ions in the body and by removing undesirable metal ions where chelators bind with metals to form complexes whereby enhancing their excretion out from body (Gómez *et al*., 1998; Scott *et al*., 2009; Tubafard *et al*., 2010; Saljooghi *et al*., 2010; 2011; Sears *et al*., 2013). In lead poisoned humans, sodium citrate proved to be effective in reducing the lead levels to normal or near normal levels (Kety and Letonoff, 1941). Additionally, combination therapy consisting of antioxidants along with chelators have been proved to be more effective in restoring antioxidants status altered by heavy metal toxicity (Flora *et al*., 2002; 2003; 2004a; 2004b; 2008; Gautam *et al*., 2010). Accompanying

antioxidants like taurine, melatonin, lipoic acid, N-acetylcysteine and gossypin along with chelators like monoisoamyl DMSA and succimer in animal experiments have proved to be beneficial in recovering from physiological damage to considerable extent.

The gut microbiome allows only 40 to 60 % of ingested metal to be transported across intestinal barrier by preventing absorption by sequestering them (Valentine *et al*., 1979; Wester *et al*., 1992; Zubero *et al*., 2010). Some species of lactic acid bacteria (LAB) including *L. rhamnosus, L. plantarum*, and *Bifidobacterium longum* are capable of binding heavy metals *in vitro* while some are known to have antioxidative properties in human subjects (Kullisaar *et al., 2*003; Halttunen *et al., 2*007; *2*008; Ejtahed*et al., 2*012), which may be considered to be an important characteristic for ameliorating heavy metal toxicity. Probiotic *E. coli* Nissle 1917 was known to impede the reactive oxygen species (Schumann *et al*., 2012). The probiotic *E. coli* CFR 16 expressing *Vitreoscilla* hemoglobin (*vgb*) and *pqq* gene not only acted as a better antioxidant but also protected against CCl_4 and dimethyl hydrazine induced liver and colon damage coupled with altered neurotransmitter status (Kumar *et al*., 2014; Pandey *et al*., 2014; 2015). Similarly, *Ec*N-4 secreting PQQ was demonstrated to be more effective than orally given PQQ against rotenone and alcohol induced oxidative stress (Singh *et al*., 2014; 2015).

Most bacteria, including gastrointestinal microbiota, do not have the property to secrete citric acid. However, overexpression of an artificial operon (*csYF-citC*) comprising of NADH insensitive *E. coli cs* Y146F mutant gene and *S. typhimurium* Na^+ dependent citrate transporter (*citC*) gene resulted into high levels of citric acid secretion up to 9 mM in many bacteria (Adhikary *et al*., 2014; Wagh *et al*., 2014; Yadav *et al*., 2014). The present study was done to evaluate the effect of *Ec*N-20 producing PQQ and *Ec*N-21 possessing artificial citrate operon (*csYF-citC*) and *pqq* gene cluster producing citric acid along with PQQ as a combination therapy involving probiotic *Ec*N, PQQ as antioxidant and citric acid as chelator against Hg induced toxicity.

4.1. Methods and Materials

4.1.1 Animals:

Male Charles foster albino rats (weight 250–300 g) were given free access to food and water and were maintained at relative humidity (45.5 %), controlled temperature (25±1 ºC), photoperiod cycle (12 h light: 12 h dark) as per recommended by Committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines of Animal Ethical Committee of the Institute (M. S. University of Baroda, India, **Reg. No. 938/A/06/CPCSEA).**

4.1.2 Construction of pJET plasmid containing *pqq* and *csYF-citC*:

Constitutive *tac* promoter (p*tac*) was obtained by amplification of pMALp2 using F1 and R1 primers (Table 4.1 and 4.2). *pqq* gene cluster amplified from the genome of *Gluconobacter oxydans* using Forward primer (F2) and Reverse primer (R2) to obtain amplicon of 3.3kbp. These amplicons were then mixed and amplified using F1 and R2 primers using recombinant PCR to generate amplicon *(*p*tac-pqq)* of 3.4 kb. Amplicon of p*tac-pqq* was then cloned into pJET generating pRN1. Artificial citrate operon (*csYF-citC)* was amplified from pJNK4 by using Forward primer F4 and Reverse primer R4 to obtain amplicon of 3.1kb and constitutive p*tac* was amplified from pMALp2 using Forward primer F1 and Reverse primer R3. Recombinant PCR using Forward primer F1 and Reverse primer R4 was performed to obtain amplicon (p*tac-csYF-citC*) of 3.2kb, which was then cloned in pJET to get pRN2 plasmid. pRN2 was digested with XhoI to release p*tac-csYF-citC* and ligated with pRN1 opened with XhoI to obtain pRN3 plasmid. This was then transformed into *Ec*N-2. *Ec*N was obtained as a generous gift from Dr. Rer. Nat. Ulrich Sonnenborn, Ardeypharm GmbH, Loerfeldstrabe 20, Herdecke (Germany). *Ec*N strain was modified with genomic integration of *vgb* and *gfp* genes to produce *Ec*N-2 (Singh *et al*., 2014).

F1	CCCTCGAGGGTTGACAATTAATCATCGGCTCGTATAATGGATCGAA TTGTGAG
R1	CCA GGCCAT AAT CTA TGG TCC TTG TTG GTG AAG TG
F2	CAAGGA CCATAGATT ATG GCC TGG AAC ACA CCGA
R2	CC CTCGAG GG TTA CGT ATA ACG CCT GTA GAA CAA CGT GC
R3	GCTTTTGTATCAGCCAT AAT CTA TGG TCC TTG TTG GTG AAG TG
F4	CAAGGACCATAGATT ATG GCT GAT ACA AAA GC A AAA CTC ACC CTC
R4	C GAGCTC G TTA CAC CAT CAT GCT GAA CAC GAT GC

Table 4.1: List of primers used in the study.

Plasmids/ Strain	Characteristics	References
pMALp2	p*tac*, *malE*, Amp^r	NEB
pJNK4	pUCPM18 with *E. coli* NADH insensitive citrate synthase gene (*cs*YF), citrate transporter *cit*C of *Salmonella typhimurium*; Apr, Kmr	Wagh *et al*., 2014
pJET	pJET1.2/blunt is a linearized cloning vector, Recircularized pJET expresses a lethal restriction enzyme after transformation and is not propagated, Amp^r	Thermo Scientific CloneJET PCR Cloning Kit #K1231, #K1232
pRN1	pJET harboring *Gluconobacter oxydans pqq* gene cluster (3.3 Kb) under *tac* promoter, Amp^r	This study.
pRN2	pJET harboring *csYF-citC* genes from pJNK4 under *tac* promoter, Amp^r	This study
pRN3	pJET harboring *Gluconobacter oxydans pqq* gene cluster under *tac* promoter and *csYF-citC* genes from pJNK4 under *tac* promoter	This study
*Ec*N	*Escherichia coli* Nissle 1917 (*Ec*N) (Probiotic strain)	Sonnenborn *et al*., 2009
*Ec*N-2	*Ec*N strain with genomic integration of *vgb* and *gfp* genes	Singh *et al*., 2014
*Ec*N-20	*Ec*N-2 harbouring pJET-*tac-pqq*	This study
*Ec*N-21	*Ec*N-2 harbouring pJET-*tac-pqq-tac-cs*YF-*citC*	This study

Table 4.2: Plasmids and bacterial strains used in the study.

4.1.3 Characterization of *Ec*N transformants producing PQQ and citric acid

*Ec*N-20 and *Ec*N-21 transformants were confirmed by growing in Tris-buffered medium with methyl orange as pH indicator, appearance of red colour indicated pH drop due to gluconic acid secretion mediated by PQQ dependent glucose dehydrogenase (Gyaneshwar *et al*., 1999). Extraction of PQQ from *Ec*N transformants was performed according to Suzuki *et al*. (1990). In M9 minimal medium cells were grown overnight using glucose as carbon source. Cells were pellet down and culture supernatant was used for PQQ extraction which was incubated with 50 % acetonitrile at 65 °C for 2 h followed by centrifugation at 15,000 x g for 10 minutes, the clear supernatant thus obtained was dried. The obtained residues were dissolved in 50 % n-butanol and dried. At last, the residues were dissolved in water and filtered with 0.2 micron filter and quantified using Hitachi fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) with excitation 375 nm and emission 465 nm. PQQ concentration of unknown sample was determined by plotting standard curve using PQQ standard. 20% Liver tissue homogenate and 10 % colonic content homogenate in Phosphate buffer saline was used for PQQ extraction and quantification.

The batch culture studies were performed under aerobic conditions in M9 minimal medium using glucose as carbon source initially inoculated with cell suspensions to have 0.01-0.03 $O.D_{600nm}$. At regular intervals 1 ml samples were harvested aseptically and pH drop was taken as marker for acid production. After the media reached pH <5, samples were centrifuged at 9,200 x *g* for 1 min at 4 °C and the culture supernatants were filtered through 0.2 µm nylon membrane and used to estimate citric acid using HPLC (Buch *et al*., 2009). For HPLC analysis, the RP-18 column was operated at room temperature using 0.02 % orthophosphoric acid as mobile phase, at a flow rate of 0.8 ml min–1 and the column effluents were monitored using a UV detector at 210 nm. Organic acid quantification was done with reference to the peak areas obtained for the citric acid standard (Sigma Pvt. Ltd.).

4.1.4 Experimental Design

All rats were fed on normal pellet diet and were divided into 8 different groups (6 animals per group) as follows (Table 4.3):

Group I	Drinking water as Control group
Group II	80 ppm $HgCl_2$ in drinking water daily for 4 weeks
Group III	Hg and *Ec*N-*2* once per week
Group IV	*Ec*N-*2*, Hg and citric acid (1.2 g / kg body weight) orally once a week
Group V	*Ec*N-*2*, Hg and PQQ (10mg / kg body weight) orally once a week
Group VI	*Ec*N-*20* and Hg
Group VII	*Ec*N-*21* and Hg
Group VIII	*Ec*N-*2*, Hg, PQQ (10mg / kg body weight orally) and citric acid (1.2 g / kg body weight orally) once a week
Group IX	*Ec*N-*20*, Hg and citric acid (1.2 g / kg body weight) orally once a week

Table 4.3: Experimental design for animal experimentation.

4.1.5 Preparation of Tissue Homogenates

Firstly, Liver and Kidney tissues were washed with ice-cold saline after sacrificing rats, then homogenization were performed in ice cold Phosphate Buffer Saline while for estimating GSH levels tissues were homogenized separately in 5 % trichloroacetic acid.

4.1.6 Biochemical Assays

Catalase activity was measured by disappearance of hydrogen peroxide at 240 nm (Beers and Sizer, 1952). Superoxide dismutase (SOD) activity was determined by using pyrogallol method (Marklund and Marklund, 1974). Reduced GSH was determined by the method of Beutler *et al*. (1969). Lipid peroxidation was measured by estimating levels of Malondialdehyde (MDA) according to method described by (Buege and Aust, 1978).

4.1.7 ALT, AST, ALP, Total Bilirubin, Urea and Creatinine

ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), Total bilirubin, Urea and Creatinine in serum were measured using kit as per manufacturer protocol (Beacon Diagnostics Pvt. Ltd., Navsari, Gujarat, India).

4.1.8 Histopathological Changes

Liver and Kidney tissues were first fixed with 10 % buffered formalin, followed by staining of histological section with hematoxylin and eosin.

4.1.9 Metal Determination

Estimation of Hg was done by Atomic Absorption Spectroscopy according to protocol of Salinska *et al*. (2012). The colonic contents were collected after sacrificing rats, then for digesting tissues concentrated nitric acid was carefully added to it and heated at 65–70 ^{0}C. Perchloric acid was then added for protein precipitation. It is heated again till the fumes came out and then made up the volume to 20 ml with double distilled water. The Hg content of the samples was then measured using an atomic absorption spectrophotometer. The Hg content was expressed in mg/g of rat faeces.

4.1.10 Statistical Analysis

The statistical significance of the values was determined by one-way analysis of variance (ANOVA) using GraphPad Prism Version 5.0 (GraphPad Softwares Inc., San Diego, CA). The results were considered significant at $p \leq 0.05$.

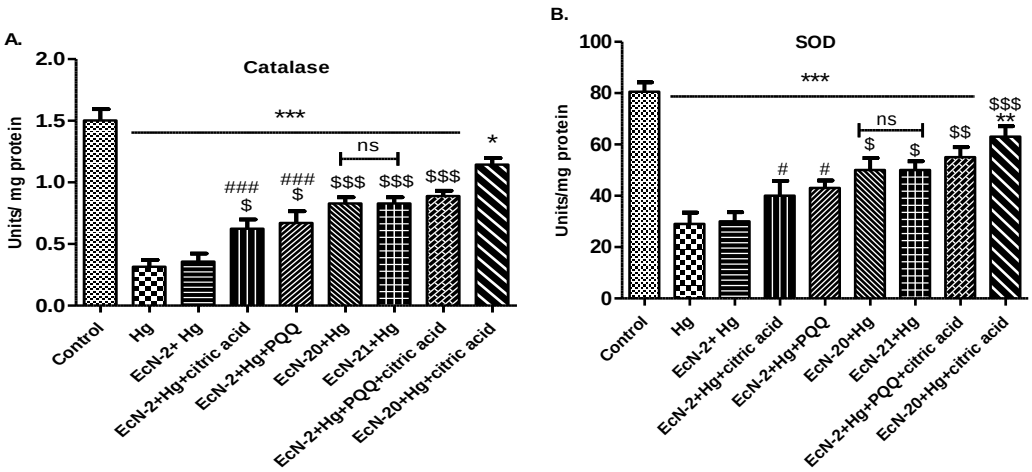
4.2 Results

4.2 .1 Characterization of *Ec*N-2 transformants

After 24 h in M9 minimal medium at 37 °C with glucose as carbon source, *Ec*N-20 and *Ec*N-21 transformants produced 5.6 ± 0.12 µg PQQ/ml of culture. Both transformants acidified Tris-buffered medium due to secretion of gluconic acid by PQQ dependent glucose dehydrogenase with methyl red as indicator. Additionally, *Ec*N-21 produced 2 ± 0.09 mM of citric acid.

4.2.2 Effect of *Ec*N probiotic against Hg induced oxidative stress in liver and Kidney in rats

Hg exposure for one month decreased the GSH levels, Catalase and SOD activity while increased the Lipid peroxidation significantly in Liver and Kidney compared to control (Fig.4.1, 4.2). However, *Ec*N-20+citric acid (oral) was most effective against Hg exposure, by increasing GSH levels, Catalase and SOD activity, in decreasing the Lipid peroxidation in liver and kidney. Nevertheless, protection shown by *Ec*N-20+citric acid (oral) was not sufficient in bringing the damage to normal levels against Hg toxicity. For Hg toxicity, *Ec*N-20 was more effective than *Ec*N-2+PQQ (oral) in increasing Catalase and SOD activity, GSH levels and in decreasing Lipid peroxidation in Liver and Kidney.



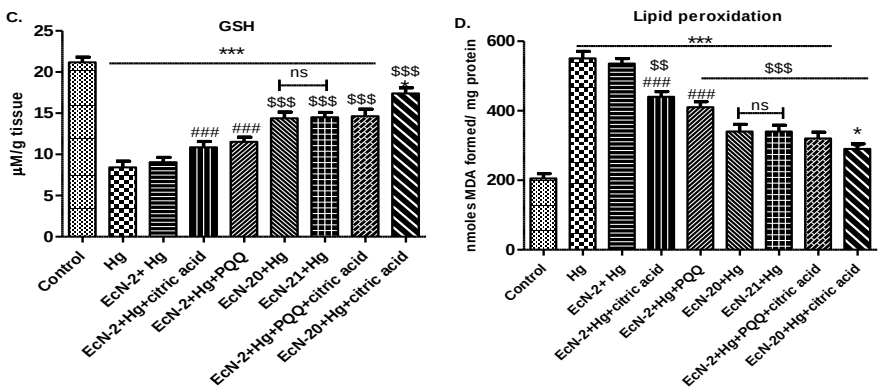


Figure 4.1: Effect of genetically engineered probiotic E. coli Nissle 1917 on antioxidant parameter in Liver on Hg exposure (A) Catalase (B) SOD activity (C) GSH levels (D) Lipid peroxidation. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, ** P ≤ 0.01 and ***P ≤ 0.001 compared to Control group. $P ≤ 0.05, $$ P ≤ 0.01, $$$P ≤ 0.001compared to Hg group, #P ≤ 0.05, ## P ≤ 0.01 and ###P ≤ 0.001 compared to EcN-20+Citric acid(oral)+Hg.

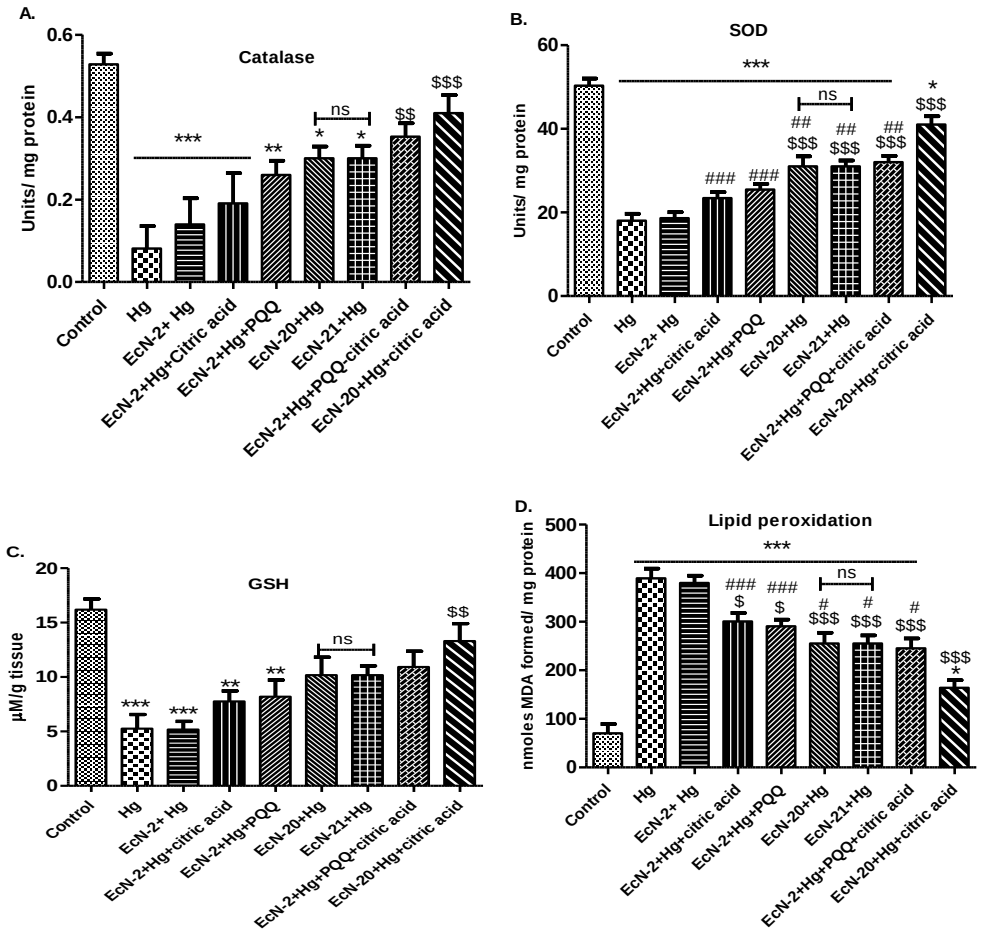


Figure 4.2: Effect of genetically engineered probiotic E. coli Nissle 1917 on antioxidant parmeter in Kidney on Hg exposure. (A)Catalase (B) SOD activity(C) GSH (D) Lipid peroxidation. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, ** P ≤ 0.01 and ***P ≤ 0.001 compared to Control group. $P ≤ 0.05, $$ P ≤ 0.01, $$$P ≤ 0.001compared to Hg group, #P ≤ 0.05, ## P ≤ 0.01 and ###P ≤ 0.001 compared to EcN-20+Citric acid(oral)+Hg.

4.2.3 Effect of *Ec*N probiotic against Hg induced liver damage marker in rats.

Hg exposure for a month significantly increased the AST, ALT and ALP activities as compared to control (Fig 4.3). *Ec*N-2 treatment had no effect in decreasing AST, ALT and ALP activities compared to *Ec*N-2+citric acid (oral), *Ec*N-2+PQQ (oral), *Ec*N-20, *Ec*N-21, *Ec*N-2+PQQ(oral)+citric acid (oral).Moreover protection shown by *Ec*N-21 found to be same as that of *Ec*N-20.

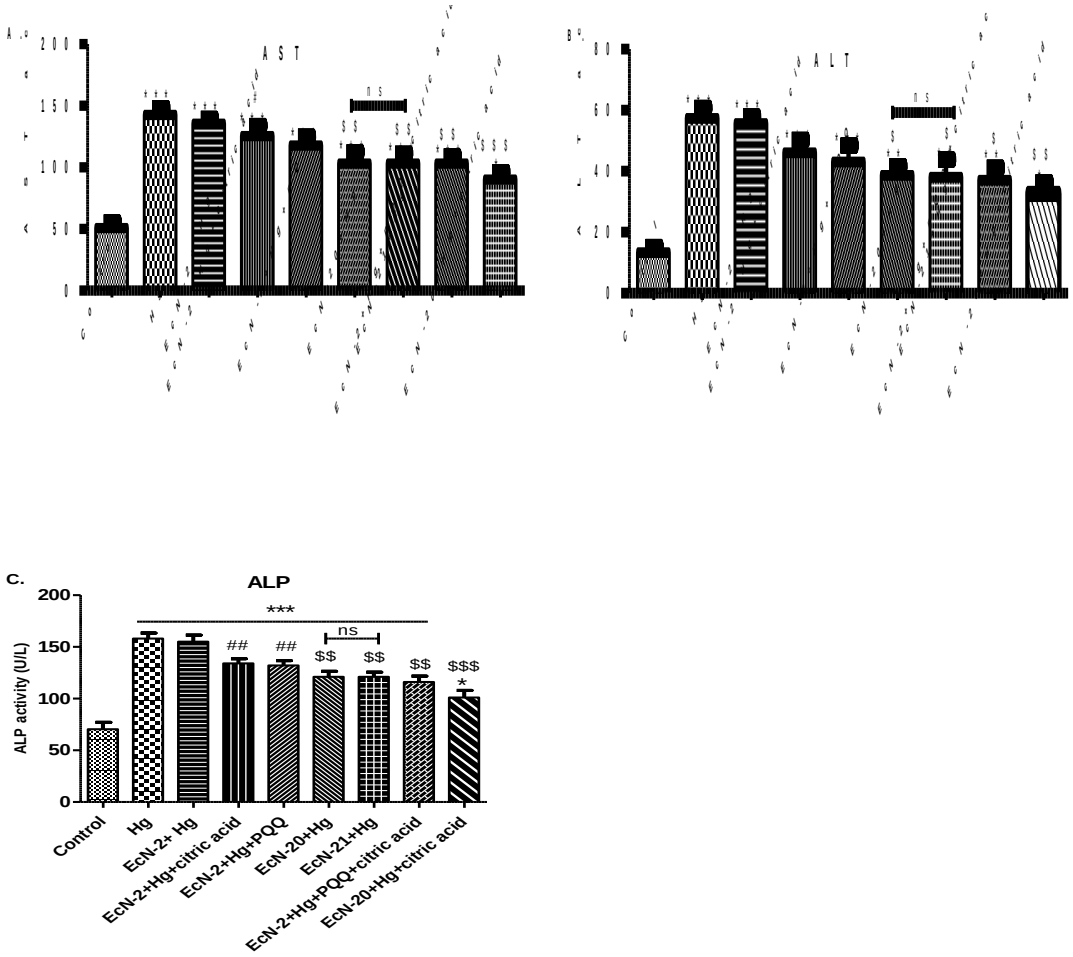


Figure 4.3: Effect of genetically engineered probiotic *E. coli* Nissle 1917 on hepatic injury marker (A) AST (b) ALT (c) ALP activity (d) Bilirubin levels in serum. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, ** P ≤ 0.01 and ***P ≤ 0.001 compared to Control group. $P ≤ 0.05, $$ P ≤ 0.01, $$$P ≤ 0.001compared to Hg group, #P ≤ 0.05, ## P ≤ 0.01 and ###P ≤ 0.001compared to *Ec*N-20+Citric acid(oral)+Hg.

4.2.4. Effect of *EcN* probiotic treatment on kidney damage marker in rats.

Hg exposure for a month also resulted in significantly increased levels of bilirubin, urea and creatinine levels in serum compared to control (Fig 4.4). *Ec*N-2 treatment had no effect in decreasing bilirubin, creatinine and urea levels in serum compared to *Ec*N-2+citric acid (oral), *Ec*N-2+PQQ (oral), *Ec*N-20, *Ec*N-21, *Ec*N-2+PQQ (oral)+citric acid (oral). Moreover protection shown by *Ec*N-21 was found to be similar to that of *Ec*N-20.

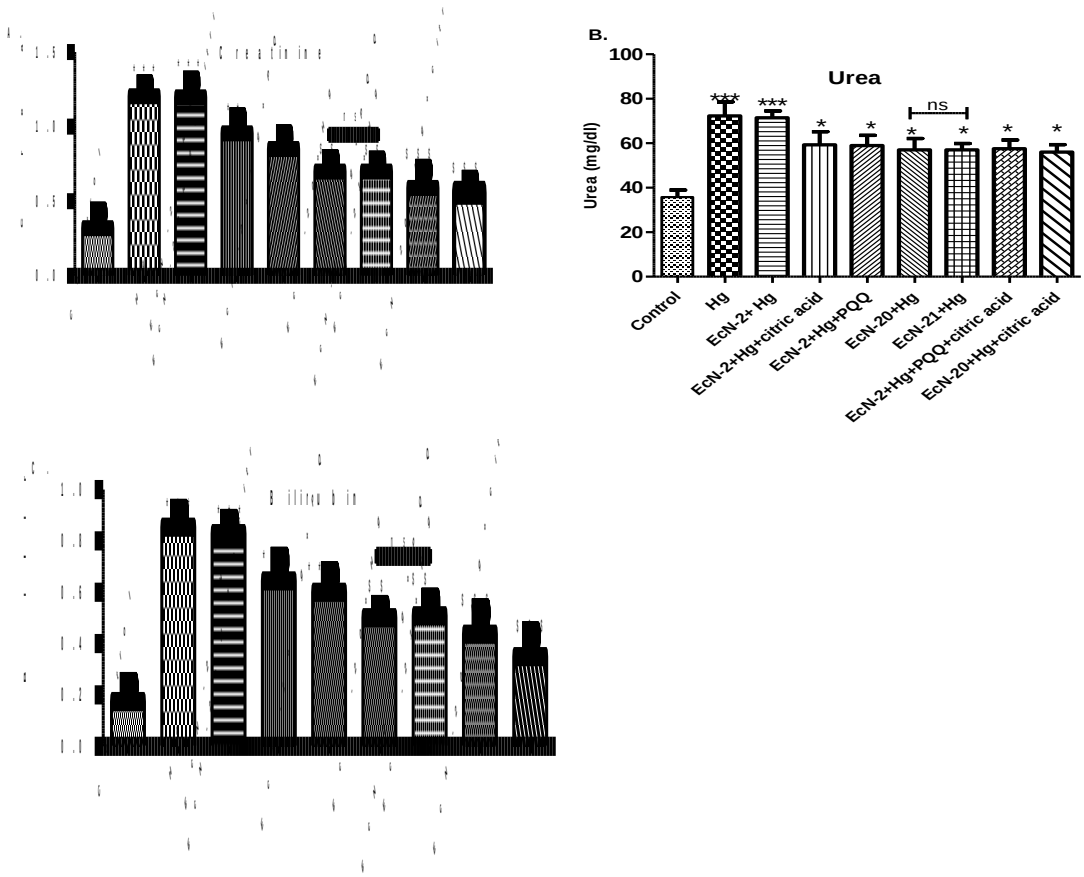


Figure 4.4: Effect of genetically engineered probiotic *E. coli* Nissle 1917 on Kidney damage marker on Hg exposure (A) Creatinine (B) Urea (C) Bilirubin levels in serum. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, ** P ≤ 0.01 and ***P ≤ 0.001 compared to Control group. $P ≤ 0.05, $$ P ≤ 0.01, $$$P ≤ 0.001compared to Hg group, #P ≤ 0.05, ## P ≤ 0.01 and ###P ≤ 0.001 compared to *Ec*N-20+Citric acid(oral)+Hg.

4.2.5 PQQ Quantification from faeces and Liver

PQQ levels were estimated from feces and Liver of rats treated with *Ec*N-20 and *Ec*N-21 (Table 4.4). In Hg exposure, PQQ levels found to be 4.65±0.24, 4.34±0.21, 4.92±0.26 nmoles/g wet wt of faeces and 232.15±7.7, 220.84±6.4, 242.62±3.9 picomoles/g Liver tissue in Group VI, VII and IX which are significantly higher compared to control and PQQ (oral) given Group (Group V).

Hg Groups	I	II	1I1	IV	V	VI	VII	VIII	IX
Fecal (n moles/ g fecalwet weight)	0.697 ±0.1	0.681 ±0.16	0.705 ±.09	0.728 ±0.11	0.785 ±0.22	4.65 ±0.24***	4.34 ±0.21***	0.773 ±0.28	4.92 ±0.26***
Liver (picomol es/ g tissue)	23.16 ±7.1	24.61 ±5.4	25.12 ±6.7	23.89 ±7.2	30.74 ±9.2	232.15 ±7.7***	220.84 ±6.4***	30.91 ±7.1	242.62 ±3.9***

Table 4.4: PQQ Concentration in fecal matter and Liver Homogenate of rats*P ≤ 0.05 compared to Group I.**

4.2.6 Hg Estimation

Levels of Hg were measured from faecal matter by Atomic Absorption Spectroscopy to determine the functionality of citric acid as chelator (Fig.4.3). Faecal Hg levels were significantly higher in Group IV, VIII, IX with orally administered citric acid as compared to Hg, *Ec*N-2+Hg, *Ec*N-2+Hg+PQQ (oral), *Ec*N-20+Hg, *Ec*N-21+Hg.

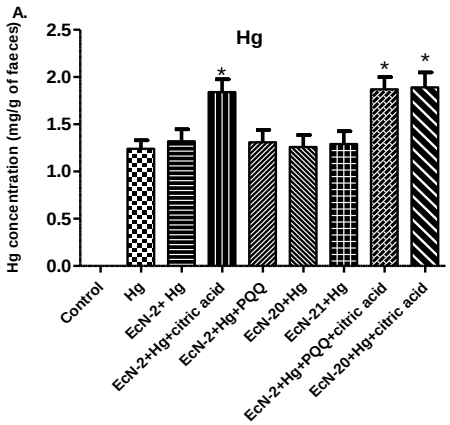
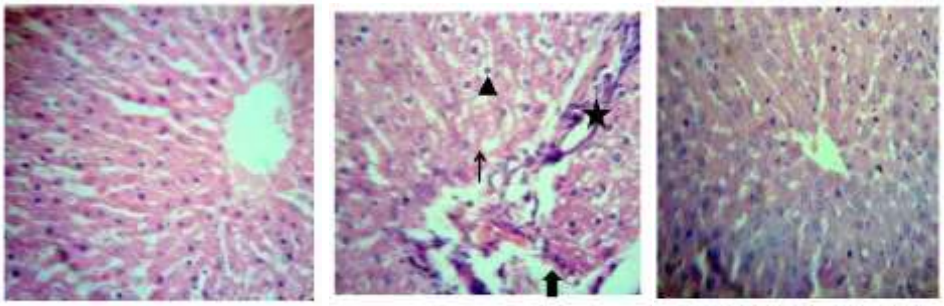


Figure 4.5:Effect of genetically engineered probiotic *E. coli* Nissle 1917 on levels of Mercury (Hg) in faeces of rats.Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05 compared to Hg, *Ec*N-2+Hg, *Ec*N-2+ Hg+PQQ (oral), *Ec*N-20+Hg, *Ec*N-21+Hg.

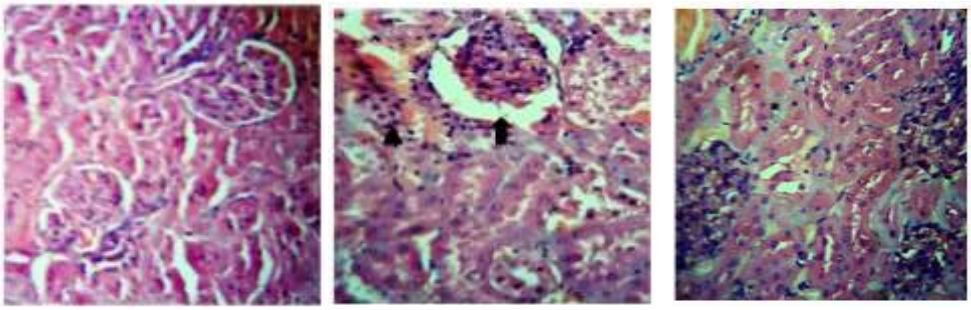
4.2.7 Histological analysis

Histological analysis showed that control group had normal architecture while introduction of Hg induced pathological changes in Liver and Kidney (Fig.4.4 and 4.5). Hg exposure caused necrosis, inflammatory cell infiltration, derangement of hepatic cords, degenerated hepatocytes, hepatic sinusoidal dilation, hepatocyte vacuolation and polymorphic nuclei in Liver while in Kidney leads to vacuolar degeneration of tubular epithelial cells, necrotic renal tubules, distention of Bowmen's cavity, Shrinkage and reducing size of glomeruli. Treatment with *Ec*N-2 resulted in no improvement in histological damage. On the other hand, treatment with *Ec*N-2 +citric acid (oral), *Ec*N-2 + PQQ (oral), *Ec*N-2+PQQ(oral)+Citric acid(oral), *Ec*N-20, *Ec*N-21 caused normal appearance of Liver and Kidney but the most effective group was *Ec*N-20 with orally given citric acid in regaining near normal appearance of Liver and Kidney.



A:Control B:Hg C:EcN-20+Citric acid)oral)+Hg

Figure 4.6: Histological sections of Liver stained with HE (Magnification=40x)(A) Control (B) Mercury treated (C) *Ec*N-20+Hg+citric acid (oral).



A:Control B:Hg C:EcN-20+Citric acid)oral)+Hg

Figure 4.7: Histological sections of Kidney stained with HE (Magnification=40x) (A) Control (B) Mercury treated (C) *Ec*N-20+Hg+citric acid (oral).

4.3 Discussion

Hg in present in regions with high levels of pollution in air, water, food or soil standing as the third most dangerous heavy metal after arsenic and lead (Emsley 2001; Othman *et al*., 2014).It is found not only in food but also in dental amalgam (Goodrich *et al*., 2016).Hg is mainly found to cause neprotoxicity and hepatoxicity (Roza *et al*., 2005; Franciscato *et al*., 2011; Moraes-Silva *et al*.,2012; Oliveira *et al*., 2014; Deng *et al*., 2014; Karapehlivan *et al*., 2014; Ansar and Iqbal, 2016). Unbound metal form increases once MT gets saturated and causes subsequent damage after MT forms complex with heavy metals decreasing their body burden (Garcia-Nino *et al*., 2014). Heavy metal toxicity is mediated by oxidative stress with higher reactivity for sulfhydryl groups located at the enzymes active site leading to covalent attachment with sulfhydryl groups of proteins including antioxidant enzymes and tripeptide GSH, thereby decreasing their levels (Bondy *et al*., 1996; Quig *et al*., 1998; Hultberg *et al*., 2001) or by replacing cofactor of these enzymes (Donaldson *et al*., 1991). Heavy metal could replace iron and copper from ceruloplasmin and ferritin, leading to increase in total bulk of free iron and copper ions, thus generating free radicals via the Fenton reactions (Waisberg *et al*., 2003). ROS induced tissue damage can also be illustrated by oxidative degradation of polyunsaturated fatty acids within biological membranes leading to lipid peroxidation. Mercuric Chloride treated mice showed alterations in the lipid peroxidation, glutathione reductase, glutathione peroxidase, superoxide dismutase and GSH levels (Mahboob *et al*., 2001). Glomerular filtration has been shown to be reduced by tubular injury induced by ROS causing contraction of mesangial cells with changes in ultrafiltration coefficient factors and surface area for filtration, thus ultimately increasing urea and creatinine levels in blood (Necib *et al*., 2013).

Elevated levels of AST, ALT, ALP and bilirubin are the markers for acute Liver damage. Oxidative damage changed the liver permeability causing leakage of these enzymes into bloodstream (Naik *et al*, 2011; Kang *et al*., 2013). Present study found significant damage to liver and kidney due to Hg. The 10^{14} bacterial cells present in gut has been proposed to confer protection by several resistance mechanisms such as by sequestering metals, transforming them to a less toxic form and actively transporting metal out of the cell (Mowll *et al*., 1984; Hamlett *et al*.,

1992; White *et al*., 1998). Even though, *Ec*N-2 was not effective in dealing with toxicity associated with Hg, *Ec*N-20 was found to be more effective than *Ec*N-2 with PQQ (oral). Similar effects of alleviation of oxidative stress induced by administration of DMH and alcohol were found with probiotic *E. coli* CFR 16::v*gb-gfp* (*pqq*) and *Ec*N secreting PQQ (Pandey *et al*., 2014; Singh *et al*., 2014).

Excitingly, present study proved that *Ec*N-20 with citric acid (oral) was most effective in dealing metal toxicity. Clinically important chelators include EDTA, DMPS, DMS, and BAL (Bernhoft *et al*., 2013). Chelators have been proposed to mobilize heavy metals from tissues to Kidney for excretion in urine and to Liver for excretion through bile (Rooney *et al*., 2007). Constitutive production of PQQ within gut by *Ec*N-20 resulted to the protection shown by *Ec*N-20+citric acid (oral). PQQ then absorbed by gut reaches to different organs like Liver and Kidney, where it scavenges free radicals produced by Hg. Application of citric acid will chelate Hg that remain free after metallothionein saturation preventing their accumulation in Liver and Kidney, thereby leading to faecal excretion of Hg. Citric acid majorly enhanced the biliary excretion of metals, thus helps in faecal excretions of metals (Kojima *et al*., 1976). *Ec*N-21 producing PQQ and citric acid was as effective as *Ec*N-20 producing PQQ alone. This could be attributed to inefficient chelation of Hg due to low levels of citric acid. PQQ produced by *Ec*N-20 and *Ec*N-21acted as a co-factor for glucose dehydrogenase. The active glucose dehydrogenase facilitated the conversion of glucose to gluconic acid, a prebiotic (Kameue *et al*., 2004), which is utilized by colonic microflora resulting into the production of SCFAs. This is supported by our previous study, where *Ec*N secreting PQQ had high levels of SCFAs like butyric and propionic acids in the feacal matter of the rats (Singh *et al*, 2014).Histopathological analysis have also shown current strategy to be more effective in healing liver induced damage as kidney is major organ affected by metal toxicity (Yang *et al*., 2015).

4.4 Conclusion:

Present study demonstrates that *Ec*N-20 producing PQQ supplemented with citric acid orally to be an effective strategy to deal with Hg induced Liver and Kidney damage compared to orally given PQQ and citric acid. *Ec*N-21 secreting PQQ and citric acid was found to have similar effects to that of *Ec*N-20 producing PQQ supplemented with citric acid orally which could be due to insufficient chelation of Hg due to low levels of secreted citric acid. More efficient probiotic secreting stronger chelating agent needs to be produced for dealing with heavy metal induced toxicity.