Chapter 5:

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

5. Introduction

Pathophysiology of metals depends primarily on the generation of oxidative stress (Jan *et al*., 2015). Mercury accumulating mainly in the kidneys shows higher binding affinity for endogenous thiol containing molecules such as glutathione and cysteine causing oxidative stress and renal damage by damaging the antioxidant defence system and depleting the thiol reserves (Hultberg *et al*., 2001; Zalups *et al*., 2000).

Antioxidants are thought to play a vital role in neutralizing free radical-induced damage to macromolecules by heavy metal exposure and have been found to repair the free radical mediated cell injury (Arslan *et al*., 2010). Dietary antioxidants, primarily free radical scavengers act through various mechanisms in different compartments by diminishing peroxidation of membrane lipids, direct neutralization of free radicals thereby decreasing ROS production and neutralizing ROS with short-chain free fatty acids and cholesterol esters through lipid metabolism. The molecular change by heavy metal exposure induced apoptosis in rats was reported to be reversed by antioxidants like Vitamin C and Vitamin E (Ramanathan *et al*., 2005). Pyrroloquinoline quinone (PQQ), a ubiquitous molecule has been shown to have varied biochemical and physiological role including powerful antioxidative, antidiabetic, neuroprotective and growth promoting effect (Akagawa *et al*., 2015). Organic acids such as 2-Ketogluconic acid (2-KG) produced by bacteria have been shown to solubilize various divalent metals (Duff *et al*., 1963). Low level of environmental exposure of toxic metals may be asymptomatic but vasculotoxic and hence increasing their excretion through chelation may prove advantageous (Aneni *et al*., 2016).Metal chelating drugs are used for the treatment of acute heavy metal exposure; however, they are not intended for long-term use and there is a lack of regulatory-approved consumer products for chelation (Bisanz *et al*., 2014). Major side effects of chelation therapy include imbalance of essential metal ion homeostasis, hepatotoxicity and nephrotoxicity (Crisponi *et al*., 2014).

When *pqq* gene clusters are incorporated in *E. coli* it resulted in PQQ biosynthesis and active glucose dehydrogenase enzyme resulting in the conversion of glucose into gluconic acid (Goosen *et al*., 1989 Khairnar *et al*., 2003). In the periplasm gluconate dehydrogenase (GADH) enzyme encoded by *gad* operon converts gluconic acid into 2-ketogluconic acid by using FAD as cofactor (Hirohide *et al*., 2007). *gad* operon of *E. cypripedii* ATCC 29267 was overexpressed in *E.coli* that enabled secretion of high amount of 2-ketogluconic acid (Yum *et al*., 1997). Several resistance mechanisms have been developed by gut microflora for prevention of heavy metal toxicity such as sequestering heavy metals on their surfaces thereby removing them through subsequent defecation or by active expulsion from cytosol (Monachese *et al*., 2012). The mercury and arsenic blood levels were found to be decreased in the pregnant women upon consumption of probiotic yogurt containing 10^{10} CFU *Lactobacillus rhamnosus* GR-1 (Bisanz *et al*., 2014).

E. coli Nissle 1917 (*Ec*N), a well-known probiotic apart from being non-invasive, nonpathogenic it does not produce enterotoxins (Sonnenborn *et al*., 2009). *Vitreoscilla* hemoglobin (*vgb*) and *pqq* genes containing probiotic *E. coli* CFR 16 have been shown to be protective against CCl_4 and dimethyl hydrazine induced liver and colon damage along with altered neurotransmitter levels (Kumar *et al.,* 2014; Pandey *et al*., 2014,2015). Probiotic *Ec*N-4 and *Ec*N-5 secreting PQQ was found to be effective against alcohol and rotenone induced oxidative stress (Singh *et al*., 2014; 2015). Additionally, supplementing citric acid along with *Ec*N-20 producing PQQ proved to be effective against Cd and Hg induced hepatotoxicity whereas *Ec*N-21 secreting both PQQ and citric acid was not as effective due to low level of citric acid (Raghuvanshi *et al*., 2015). Therefore, the present strategy was performed to evaluate the effect of *Ec*N-23 producing PQQ and 2-ketogluconic acid against Hg toxicity.

5.1. Methods and Materials

5.1.1 Animals:

Male adult Charles foster albino rats (weight 250–300 g) were allowed 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).**

5.1.2 Construction of p*tac-pqq-gad* plasmid*:*

Dr. Goosen, Molecular Genetics, University of Leiden, Netherland gifted the plasmid pSS2 containing *pqq* gene cluster of *A. calcoaceticus* while *Ec*N was obtained as a generous gift from Dr. Rer. Nat. Ulrich Sonnenborn, Ardeypharm GmbH, Loerfeldstrabe 20, Herdecke (Germany). The plasmid pSS2 was digested with *Eco*R1/ BamHI to obtain a release of 5.1 Kb of *pqq* gene cluster which was further ligated with pUCPM18 treated with *Eco*R1/ BamHI, which resulted into pJNK5. pJNK5 was further digested with BamHI/XbaI, ligated with 3.8 Kb *gad* operon of *P. putida* KT 2440 released from pCNK12 treated with BamHI/XbaI, which further resulted in pJNK6. The pJNK5 and pJNK6 were transformed into *Ec*N-2 that resulted to *Ec*N-22 and *Ec*N-23 respectively.

Strain	Characteristics	References
*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-22	*Ec*N-2 harbouring pJNK5 producing PQQ	This study
*Ec*N-23	*Ec*N-2 harbouring pJNK6 producing PQQ and 2-KGA	This study

Plasmids	Characteristics	References
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r (100µg/ml)	Hester *et al.*, 2000
pSS2	25Kb plasmid contains 5.1-kb of *pqq* gene cluster of *A. calcoaceticus* Tet^r (40µg/ml)	Goosen *et al.*, 1989
pCNK12	pJET 2.1 with *gad* operon. Amp^r (100µg/ml)	Chanchal *et al.*, 2013
pJNK5	pUCPM18, Gmr (20µg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus*.	Wagh *et al.*, 2016
pJNK6	pJNK5, Gmr (20µg/ml) with *gad* operon 3.8 Kb of *P. putida* KT 2440	Wagh *et al.*, 2016

Table 5.1: Plasmids and strains used in the study.

5.1.3 Characterization of *Ec*N transformants producing PQQ and 2-ketogluconic acid

*Ec*N-22 and *Ec*N-23 transformants were confirmed by growing in Tris-buffered medium with methyl orange as pH indicator. PQQ dependent glucose dehydrogenase and FAD dependent gluconate dehydrogenase resulted into formation of gluconic acid and 2-ketogluconic acid, respectively. The secreted acid resulted into red colour formation indicating pH drop (Gyaneshwar *et al.*, 1999). Extraction of PQQ from *Ec*N transformants was carried out according to (Suzuki *et al.*, 1990). Cells were grown overnight in M9 minimal medium using glucose as carbon source. The culture supernatant obtained after pelleting down the cells was used for PQQ extraction. The obtained supernatant 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 was then dried and the obtained residues were dissolved in 50 % n-butanol which was further dried. At last, the residues were dissolved in water and filtered with 0.2 micron filter and quantified by 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.

Estimation of short chain fatty acids in colonic content and 2-ketogluconic acid from *Ec*N transformants was carried out by HPLC analysis according to (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.

5.1.4 Bacterial Strains and Culture Conditions

*Ec*N-2, *Ec*N-22, *Ec*N-23 probiotic strains were grown overnight in Luria Broth (LB) at 37 °C to achieve final colony forming unit (CFU) of 10^9 cells/ml culture. One milliliter of this culture was then pelleted followed by washing twice and dissolving in saline which further tube fed to rats.

5.1.5 Experimental Design

All rats were fed normal pellet diet and divided into six different groups (6 animals per group) as follows (Table 5.2):

Group I	Drinking water as Control group
Group II	80 ppm Hg in drinking water daily for 4 weeks
Group III	Hg and *EcN-2* once per week
Group IV	*Ec*N-2, Hg , 2-KG (1.2 g / kg body weight) and PQQ (10mg / kg body weight)orally once a week
Group V	*Ec*N-*22* and Hg
Group VI	*Ec*N-*23* and Hg

Table 5.2: Experimental design for animal experimentation.

Rats were given streptomycin wash (5 g/l for 24 hours) followed by orally feeding probiotics dissolved in saline (10^9 CFU/rat) for 3 consecutive days. The colonization of these probiotics was confirmed after 7 days of treatment by fecal count of GFP expressing colonies. Hg (80ppm) was then given in drinking water for 4 weeks. Probiotic treatments were given once per week for 4 weeks. Orally PQQ (10mg / kg body weight) and 2-KG (1.2g / kg body weight) were given once a week.

5.1.6 Preparation of Tissue Homogenates

Liver and Kidney tissues obtained after sacrificing the rats were washed with ice-cold saline followed by homogenization in ice cold Phosphate Buffer Saline. For GSH levels estimation, tissues were homogenized separately in 5 % trichloroacetic acid.

5.1.7 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).

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

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

5.1.9 Histopathological Changes

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

5.1.10 Metal Determination

Estimation of Hg from colonic contents was done by Atomic Absorption Spectroscopy according to protocol of Salinska *et al.* (2012). The Hg content was expressed in mg/g of rat faeces.

5.1.11 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 $p \leq 0.05$.

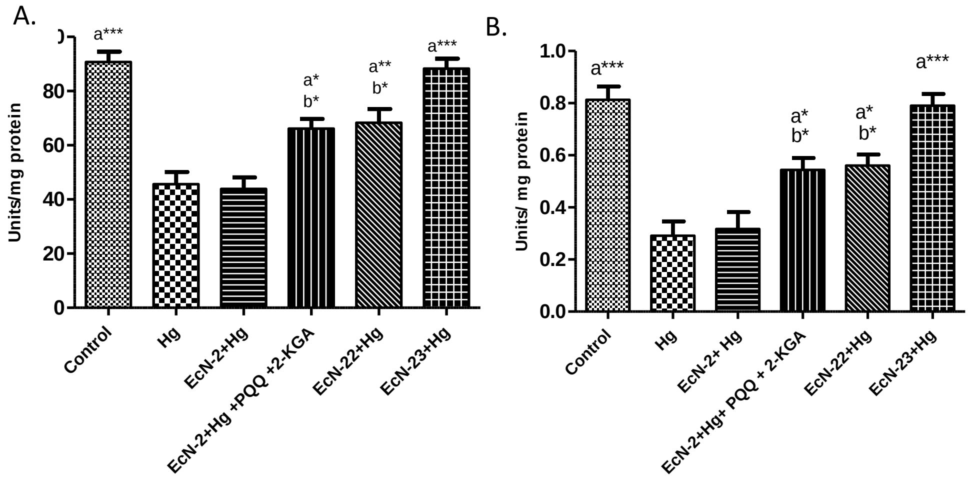
5.2 Results

5.2.1 Characterization of *Ec*N-22 and *Ec*N-23 transformants

The *Ec*N-22 and *Ec*N-23 transformants incubated for 24 h in M9 minimal medium at 37 °C produced 1.8±0.38 µg /ml and 1.6 ± 0.24 µg /ml of PQQ respectively in culture. Both transformants acidified Tris-buffered medium as seen by red coloration of methyl red indicator (Data not shown). *Ec*N-22 and *Ec*N-23 secreted 15.28±0.63mM and 5.34±0.42 mM gluconic acid while *Ec*N-23 secreted 8.16±0.19 mM 2-ketogluconic acid.

5.2.2 Effect of *Ec*N-2 transformants 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. However, *Ec*N-23 producing PQQ and 2-ketogluconic acid was most effective against Hg exposure by increasing GSH levels, Catalase and SOD activity, in decreasing the Lipid peroxidation in Liver and Kidney compared to *Ec*N-22, *Ec*N-2+PQQ (oral)+ 2-ketogluconic acid (oral).



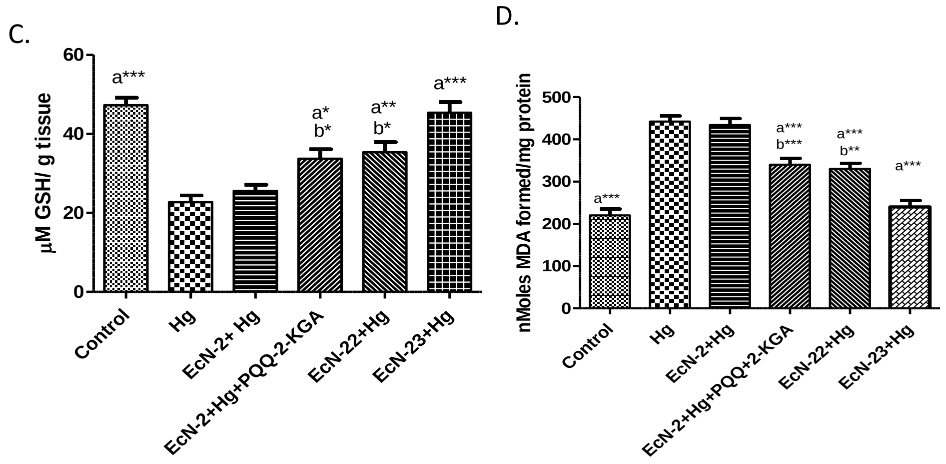


Figure 5.1: Effect of genetically engineered probiotic *E. coli* Nissle 1917 on antioxidant parameter in Liver on Hg exposure. (A)Catalase (B) SOD activity (C) GSH levels (D) Lipid peroxidation levels. Values are expressed as mean ± SEM (n=6 each group).a*p ≤ 0.05, ap ≤ 0.01, a***p ≤ 0.001 compared to Hg, b*p ≤ 0.05, b**p ≤ 0.01, b***p ≤ 0.001 compared to *Ec*N-23**

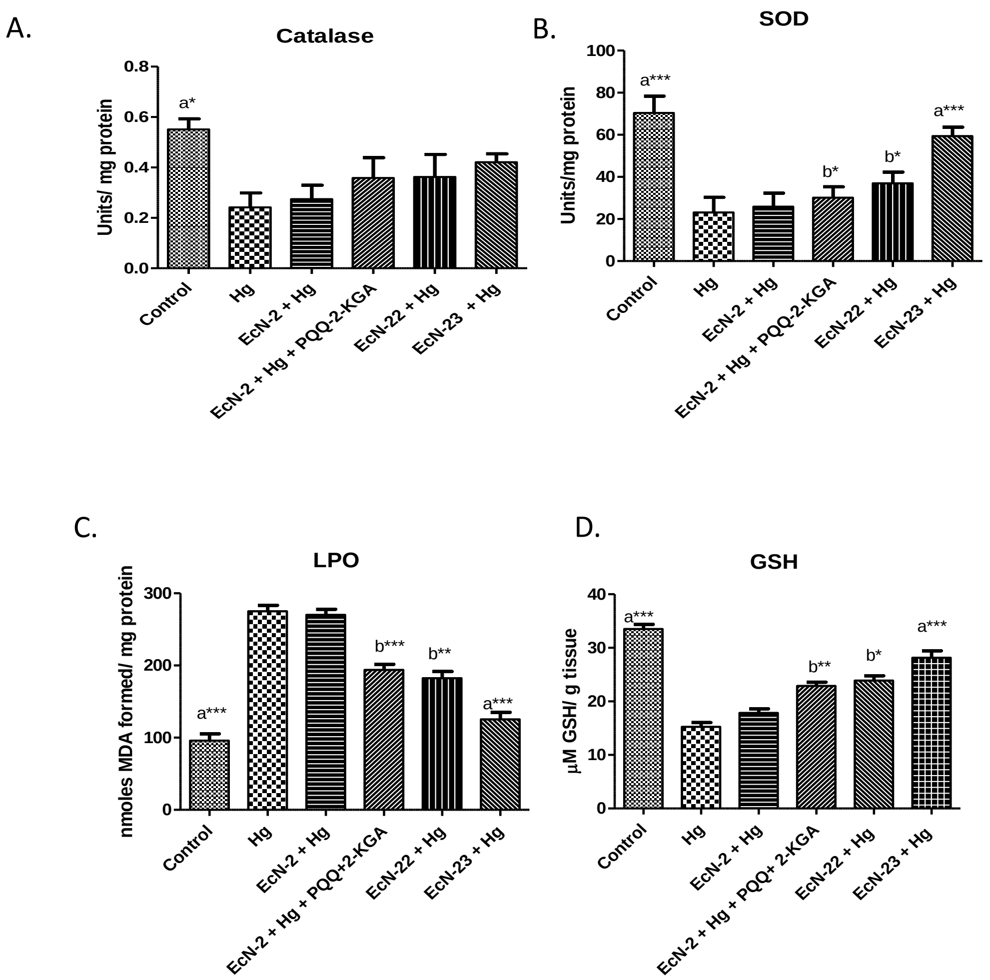


Figure 5.2: Effect of genetically engineered probiotic *E. coli* Nissle 1917 on antioxidant parameter in kidney on Hg exposure (A) Catalase (B) SOD activity (C) Lipid peroxidation (D) GSH levels. a*p ≤ 0.05, ap ≤ 0.01, a***p ≤ 0.001 compared to Hg , b*p ≤ 0.05, b**p ≤ 0.01, b***p ≤ 0.001 compared to *Ec*N-23.**

5.2.3 Effect of *Ec*N probiotic against Hg induced kidney damage in rats.

Hg exposure also increased the urea and creatinine levels significantly in serum compared to control while *Ec*N-2 treatment had no effect (Fig.5.3). *Ec*N-23 was most effective in normalizing the levels back to that of normal as compared to *Ec*N-22, *Ec*N-2+PQQ (oral) + 2-ketogluconic acid (oral).

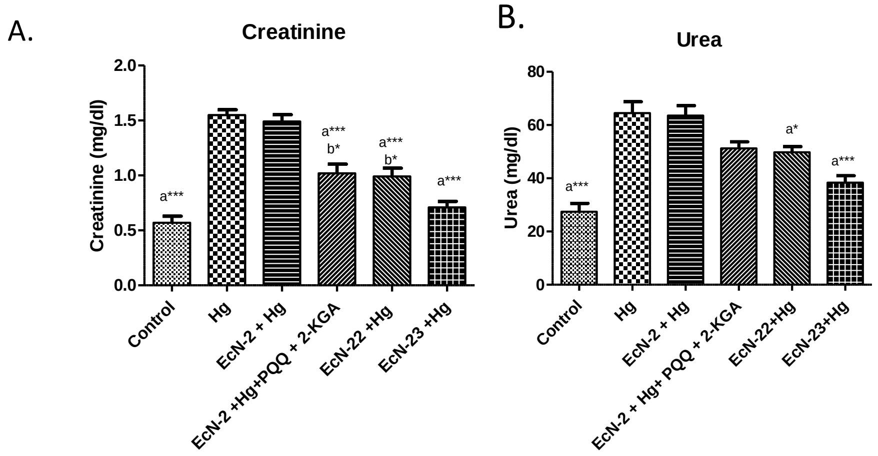
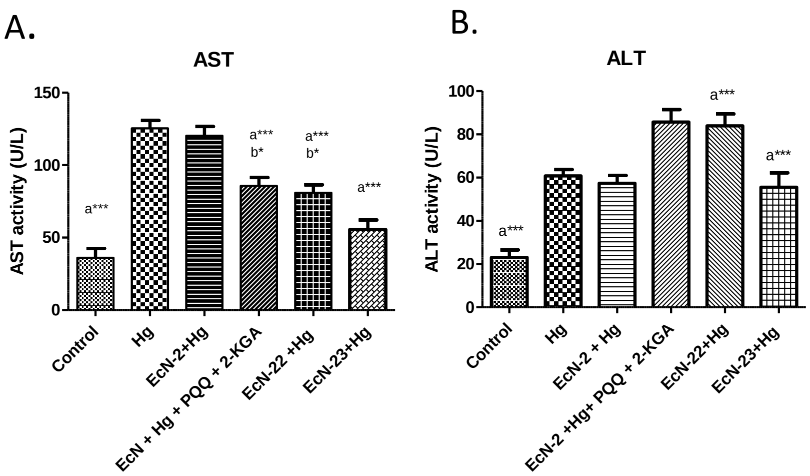


Figure 5.3: Effect of genetically engineered probiotic *E. coli* Nissle 1917 on kidney damage marker on Hg exposure (A) Creatinine (B) Urea levels in serum. Values are expressed as mean ± SEM (n=6 each group).a*p ≤ 0.05, ap ≤ 0.01, a***p ≤ 0.001 compared to Hg, b*p ≤ 0.05, b**p ≤ 0.01, b***p ≤ 0.001 compared to *Ec*N-23.**

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

Hg exposure also increased the AST, ALT and ALP activities significantly compared to control (Fig.5.4). Here also, *Ec*N-2 treatment had no effect and *Ec*N-23 could efficiently restore the levels of AST, ALT and ALP similar to that of control rats compared to *Ec*N-22, *Ec*N-2+PQQ (oral) + 2-ketogluconic acid (oral).



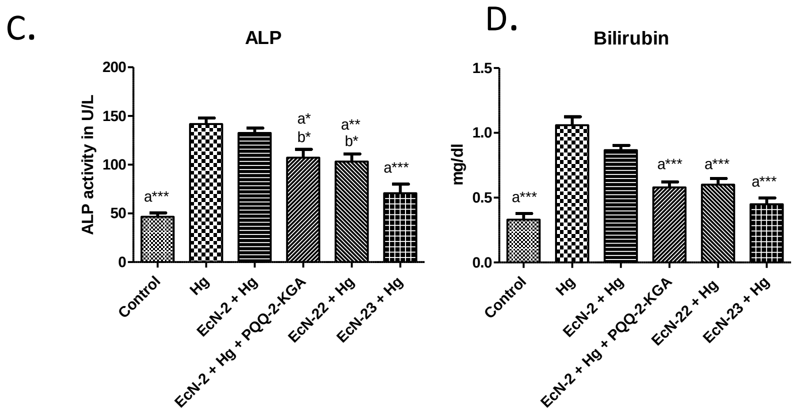


Figure 5.4: Effect of genetically engineered probiotic *E. coli* Nissle 1917 on Liver injury marker on Hg exposure. (A) AST (B) ALT (C) ALP activity (D) Bilirubin levels in serum. Values are expressed as mean ± SEM (n=6 each group).a*p ≤ 0.05, ap ≤ 0.01, a***p ≤ 0.001 compared to Hg, b*p ≤ 0.05, b**p ≤ 0.01, b***p ≤ 0.001 compared to *Ec*N-23**

5.2.5 PQQ and 2-KGA quantification from fecal matter, liver homogenate and serum

PQQ levels were estimated from feces and Liver of rats treated with *Ec*N-22 and *Ec*N-23 (Table 5.3). PQQ levels found to be significantly higher in probiotic *Ec*N-22 and *Ec*N-23 treated group as compared to control and *Ec*N-2 fed group.2-KGA levels were found to be significantly higher in *Ec*N-23 supplemented group as compared to control group (Table 5.4).

Hg Groups	Control	Hg	EcN-2	EcN-22	EcN-23
Feacal(nmoles/ g fecalwet weight)	0.681±0.16	0 .624±0.16	0.694±.09	1.748±0.11***	1.623 ±0.22***
Liver(picomoles/g tissue)	27.61±4.5	25.42±3.4	28.42±5.7	110.89±3.2***	115.34±7.2***

Table 5.3: PQQ Concentration in fecal matter and Liver Homogenate of rats. ***P ≤ 0.05 compared to Control.

Hg Groups	Control	Hg	EcN-2	EcN-22	EcN-23
Fecal (µmoles/g fecal wet weight)	nd	nd	nd	nd	192±6.7
Serum (ng/ml)	nd	nd	nd	nd	0.63±0.6

Table 5.4: 2-Ketogluconic acid (2-KG) concentration in fecal matter and serum of rats.

5.2.6 Hg Estimation

Levels of Hg were measured from faecal matter by Atomic Absorption Spectroscopy to determine the functionality of 2-ketogluconic acid as chelator (Fig. 5.5). Faecal Hg levels were significantly higher in Group V (*Ec*N-23+Hg) as compared to Hg, *Ec*N-2+Hg, *Ec*N-22+Hg.

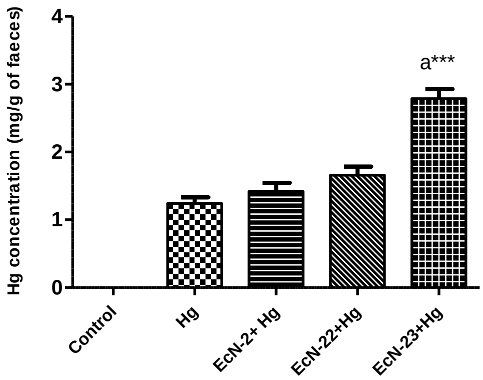


Figure 5.5: Effect of genetically engineered probiotic *E. coli* Nissle 1917 on levels of mercury (Hg) in faeces of rats. Values are expressed as mean ± SEM (n=6 each group). a*p ≤ 0.001 compared to Hg .**

5.2.7 Histological analysis

Histological analysis showed normal tissue architecture in control group whereas mercury treated group showed significant damage in liver and kidney sections. Treatment with probiotic had marked improvement in restoration of the normal architecture of kidney and liver (Fig. 5.6, 5.7).

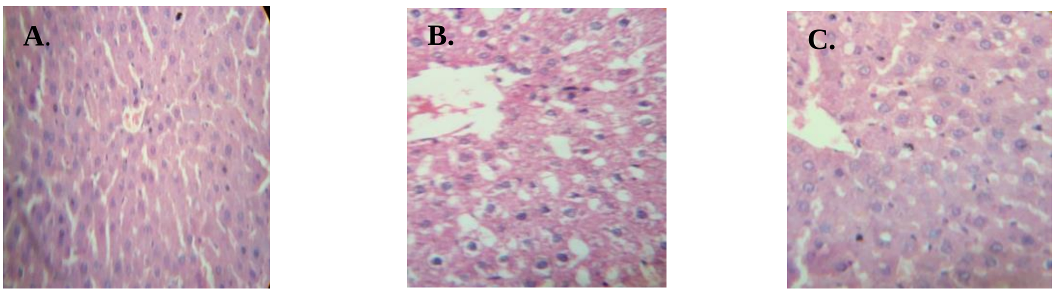


Figure 5.6 : Histological sections of Liver stained with HE (Magnification=40x) (A) Control (B) Mercury treated (C) *Ec*N-23+Hg.

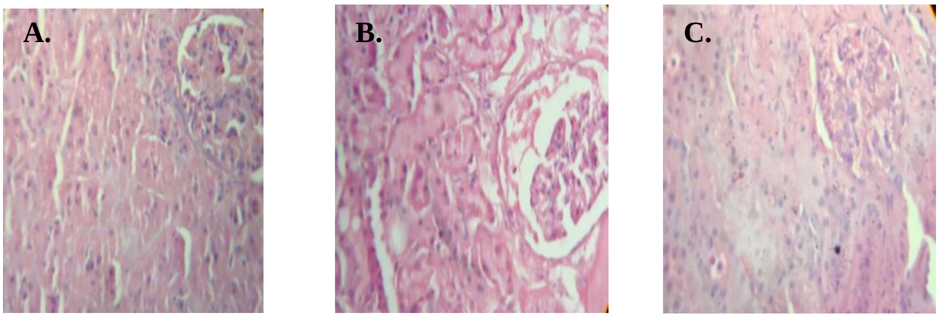


Figure 5.7: Histological sections of Kidney stained with HE (Magnification=40x) (A) Control (B) Mercury treated (C) *Ec*N-23+Hg.

5.1 Discussion

Mercury impairs the function of various organs including brain, kidney, muscles and nerves mostly through oxidative stress (Patrick, 2002). Additionally, it also damages the tertiary and quaternary structure of proteins and alters the cellular function by attaching to the selenohydryl and sulfhydryl groups. Metals can form bonds with several enzyme systems responsible for detoxifying ROS such as glutathione, depleting them and promoting accumulation of ROS and free oxygen radicals (Solenkova *et al*., 2014). Mercuric chloride treatment has been shown to increase levels of MDA along with altered SOD and catalase activities (Apaydın *et al*., 2016). Present study found significant damage to liver and kidney due to Hg as depicted by altered levels of antioxidant enzyme activities increased levels of LPO on Hg exposure. Mercuric chloride being nephrotoxic leads increase in renal damage markers, urea and creatinine in rats. Similar results were obtained in our current study. Mercuric has been demonstrated to cause cause hepatoxicity since mercuric chloride treated mice showed elevated, ALT and AST activities (Kumar *et al*., 2005). We also found elevated levels of liver function marker in this study.

Supplementing cheating agents along with antioxidant has been proved to effective strategy for heavy metal toxicity (Flora, 2002; Pande *et al*., 2002).Several studies have confirmed that probiotics have good antioxidative properties along with the capacity to uptake heavy metal from surrounding ambience (Kullisaar *et al*.,2003; Ejtahed *et al*.,2012; Bhakta *et al*.,2012) . Probiotic *E. coli* CFR 16::v*gb-gfp* (*pqq*) and *Ec*N secreting PQQ was effective against oxidative stress induced by administration of DMH, alcohol and rotenone (Pandey *et al*., 2014; Singh *et al*., 2014; 2015). Previous study demonstrated that *Ec*N-21 producing PQQ and citric acid was not as effective as *Ec*N-20 supplemented with citric acid orally against Cd and Hg induced liver and kidney damage due to low levels of secreted citric acid (Raghuvanshi *et al*., 2016). *Ec*N-23 producing PQQ and 2-ketogluconic acid was effective in reducing oxidative damage in liver and kidney induced by Hg as compared to that of orally supplemented PQQ and 2-ketogluconic acid.

5.4 Conclusion:

The present study proved that *Ec*N-23 producing PQQ and 2-KG to be more effective strategy as compared to combination therapy involving orally given PQQ and 2-KG against Hg induced toxicity.