Chapter 2

Evaluating the efficacy of probiotic *Escherichia coli* Nissle 1917 strain containing NADH insensitive citrate synthase-sodium dependent citrate transporter (*csYF-citC*) and pyrroloquinoline quinone (*pqq*) gene cluster in amelioration of cadmium induced toxicity in rats.

2.1. Introduction

The most sensitive organs to the injurious effects of environmental heavy metal pollutant like cadmium (Cd) are liver and kidney (Zalups et al., 2003). Oxidative stress mainly responsible for heavy metal pathogenesis. Cd has high affinity for thiol groups causing inactivation of thiol containing antioxidants and enzymes by binding them (Ercal et al., 2001). Since bioremediation processes as well as physical and chemical methods for heavy metal detoxification are either too expensive or ineffective (Monachese et al., 2012). Antioxidants like vitamin-C, vitamin-E, carotenoids, selenium have reported to be effective against heavy metal induced oxidative stress (Patra et al., 2011). Pyrroloquinoline quinone (PQQ), a potent antioxidant molecule, more efficient than α-tocopherol and Vitamin C due to 20,000 redox catalytic cycles (Rucker et al., 2009). PQQ also promotes mitochondrial function and biogenesis, acts as a growth factor, enhances reproductive capabilities, maintains neuronal functions (Debray et al., 2008; Rucker et al., 2009; Harris et al., 2013), reduces inflammation and liver fibrosis (Yang et al., 2014; Jia et al., 2015).

Chelation therapy is one of the widely used strategy for metal toxicity. Chelators bind with metals and enhance their excretion from body (Sears et al., 2013). In Pb poisioned humans sodium citrate was found to be effective in reducing the Pb levels near to normal (Kety et al., 1941). Combination therapy involving administration of antioxidants with chelators found to be more effective in restabilishing the tainted antioxidants status (Flora et al., 2002; 2003; 2004b; 2008; Gautam et al., 2010). Animal experiments have indicated the physiological recoveries by use of combination therapy involving chelators like monoisoamyl DMSA (meso 2,3-dimercaptosuccinic acid) and succimer with antioxidants like taurine, melatonin, lipoic acid, N-acetylcysteine and gossypin.

The gastrointestinal microbiota prevents the absorption of ingested metal by binding and sequestering them on their cell membranes (Valentine et al., 1979; Wester et al., 1992: Zubero et al., 2010). *Ec*N is non-pathogenic, non-invasive, does not produce cytotoxins or enterotoxins and found to be therapeutically effective against diarrhea, ulcerative colitis and chronic constipation (Sonnenborn and Schulze, 2009). *Ec*N was also

known to hinder the reactive oxygen species (Schumann et al., 2012). From previous laboratory reports, probiotic *E. coli* CFR 16 expressing *Vitreoscilla* hemoglobin (*vgb*) and *pqq* genes not only acted as an antioxidant but protected against CCl_4 and dimethyl hydrazine induced liver and colon damage and also prevented altered neurotransmitter status (Kumar et al., 2014; Pandey et al., 2014; 2015). Moreover, *Ec*N producing PQQ found to be more effective than orally given PQQ against alcohol and rotenone induced oxidative stress (Singh et al., 2014; 2015).

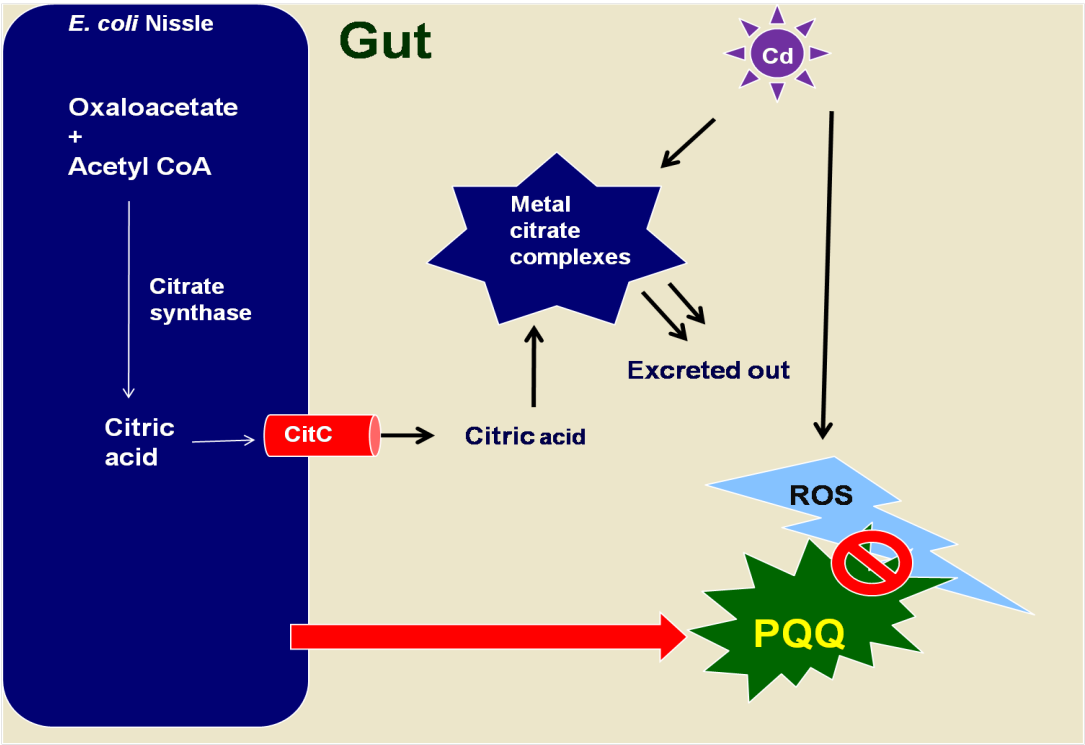

Fig. 2.1. Proposed mechanism for the detoxification of Cd using *Ec*N-20 and *Ec*N-21.

In general, gut microbiota are not known to produce citric acid. Nevertheless, upto 9 mM of citric acid was obtained in various bacteria by overexpression of an artificial operon (*csYF-citC*) consisting of NADH insensitive *E. coli cs* Y146F mutant gene along with *S. typhimurium* Na^+ dependent citrate transporter (*citC*) gene (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 with orally supplemented citric acid and *Ec*N-21 possessing artificial citrate operon (*csYF-citC*) and *pqq* gene cluster producing citric acid along with

PQQ against Cd induced hepatotoxicity and nephrotoxicity and the current hypothesis is summarized in **Fig. 2.1**.

2.2. Methods and materials

2.2.1. Animals

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

2.2.2. Cloning

Using forward primer (F1) and reverse primer (R1) constitutive *tac* promoter (p*tac*) was amplified from pMALp2 (**Table 2.1 and 2.2**). Using forward primer (F2) and reverse primer (R2) *pqq* gene cluster was amplified from the genome of *Gluconobacter oxydans* to obtain amplicon of 3.3kbp. With F1 and R2 primers amplicons of p*tac* and *pqq* gene cluster having a common overlapping regions of 24 bps were used for recombinant PCR to generate p*tac-pqq* amplicon of 3.4 kb which was then cloned into pJET to get pRN1 (**Fig. 2.2**). Using forward primer (F4) and reverse primer (R4) artificial citrate operon (*csYF-citC)* of 3.1kb was amplified from pJNK4. Using Forward primer F3 and Reverse primer R3 constitutive p*tac* amplicon was obtained from pMALp2. Constitutive p*tac* and *csYF-citC* amplicons have overlapping regions of 32 bps were used for recombinant PCR using forward primer (F3) and reverse primer (R4) to obtain p*tac-csYF-citC* of 3.2kb, which was then cloned in pJET to get pRN2 plasmid (**Fig. 2.3**). XhoI was used to digest pRN1 plasmid to release p*tac-pqq* which was ligated with XhoI digested linear pRN2 plasmid to get pRN3 plasmid (**Fig. 2.3**). *Ec*N-20 was obtained by transforming pRN1 into *Ec*N-2 while *Ec*N-21 by transforming pRN3 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).

Genes		
tac promoter	**F1 (forward)**	CCCTCGAGGGTTGACAATTAATCATCGGCTCGTATAATGGATCG AAT TGT GAG
	R1(reverse) (overlapping region of pqq gene cluster)	CCA GGCCAT AAT CTA TGG TCC TTG TTG GTG AAG TG
	F3(forward)	C GAGCTC G TTGACAATTA ATC ATC GGC TCGTATAATG GATCG AAT TGT GAG
	R3 (reverse) (overlapping region of csYF-citC)	GCTTTTGTATCAGCCAT AAT CTA TGG TCC TTG TTG GTG AAG TG
pqq (pyrroloquinoline quinone)	**F2 (forward)**	CAAGGA CCATAGATT ATG GCC TGG AAC ACA CCG A
	R2 (reverse)	CC CTCGAG GG TTA CGT ATA ACG CCT GTA GAA CAA CGT GC
csYF-citC (Artificial citrate operon)	**F4 (forward)**	CAAGGACCATAGATT ATG GCT GAT ACA AAA GC A AAA CTC ACC CTC
	R4 (reverse)	C GAGCTC G TTA CAC CAT CAT GCT GAA CAC GAT GC

Table 2.1. Primers

Plasmids/ Strain	Characteristics	References
pMALp2	P*tac*, *malE*, Amp^r	NEB
pJNK4	pUCPM18 with *E. coli* NADH insensitive citrate synthase gene (*csYF*), citrate transporter *citC* of *Salmonella typhimurium*; Amp^r, Kam^r	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 oxydens *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 oxydens *pqq* gene cluster under *tac* promoter and *csYF-citC* genes from pJNK4 under *tac* promoter, Amp^r	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* producing PQQ	This study
***Ec*N-21**	*Ec*N-2 harbouring pJET-*tac-pqq-tac-csYF-citC* producing PQQ and citric acid	This study

Table 2.2. Plasmids and bacterial strains

2.2.3. Characterization of *Ec*N transformants producing PQQ and citric acid

Confirmation of *Ec*N-20 and *Ec*N-21 transformants was done by growing them in Tris-buffered medium with methyl orange as pH indicator. pH drop indicated by appearance of red colour 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) and quantified using Hitachi fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) with excitation 375 nm and emission 465 nm. For PQQ extraction and quantification 20% liver tissue homogenate and 10% colonic content homogenate prepared in Phosphate buffer saline was used. Citric acid excreted by *Ec*N transformants was determined by HPLC analysis according to Buch et al. (2009).

2.2.4. Bacterial strains and culture conditions

Probiotic strains including *Ec*N-2, *Ec*N-20, *Ec*N-21 were grown in luria broth overnight at 37°C. Then reinoculated in fresh medium to achieve colony forming unit (CFU) of 10^9 cells/ml culture. One ml of the culture was pellet down, washed twice with saline, redissolved into saline and eventually tube fed to rats.

2.2.5. Experimental design

All rats were fed on normal pellet diet and divided into 9 different groups (6 rats per group) as follows (**Table 2.3**).

Group I	drinking water as Control group
Group II	100 ppm Cd in drinking water daily for 4 weeks
Group III	Cd and 10^9 colony-forming units (CFUs) of *Ec*N-2/rat for 3 consecutive days following streptomycin wash (5g/l for 24 hours) dissolved in sterile normal saline once a week.
Group IV	*EcN-2*, Cd and citric acid (1.2g/kg body weight) orally once a week
Group V	*EcN-2*, Cd and PQQ (10mg/kg body weight) orally once a week
Group VI	*EcN-20* and Cd
Group VII	*EcN-21* and Cd
Group VIII	*EcN-2*, Cd, PQQ (10mg/kg body weight orally) and citric acid (1.2g/kg body weight orally) once a week
Group IX	*EcN-20*,Cd and citric acid (1.2g/kg body weight orally) once a week

Table 2. 3. Experimental design for animal experiment

2.2.6. Preparation of tissue homogenates

Tissues were washed with ice-cold saline after sacrificing rats followed by homogenization in ice cold Phosphate Buffer Saline, however, for estimating GSH levels homogenization was performed in 5% trichloroacetic acid.

2.2.7. Biochemical assays

Catalase activity was determined by protocol of Beers and Sizer (1952). Superoxide dismutase (SOD) activity was determined by method of Marklund and Marklund (1974). Reduced GSH was determined by the method of Beutler et al. (1963). Lipid peroxidation (LPO) was measured by estimating the levels of Malondialdehyde (MDA) according to the method described by Buege and Aust (1978).

2.2.8. 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).

2.2.9. Histopathological changes

Firstly, tissues were fixed using 10% buffered formalin followed by dehydrated in ascending grades of ethyl alcohol. Thereafter, tissues were cleared in xylene and then mounted in molten paraplast 58-62°C. Histological sections of 5μm were cut and stained with hematoxylin and eosin before examining under bright field light microscope.

2.2.10. Cd levels

Estimation of Cd was done from colonic contents by Atomic Absorption Spectroscopy in accordance with the protocol described in Salinska et al., 2012. The content of Cd was expressed in mg/g of rat faeces.

2.2.11. Statistical analysis

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

2.3. Results

2.3.1. Cloning and characterization of *Ec*N-2 transformants

pRN1 was confirmed by PCR of *tac-pqq* using primers F1 and R2 to give amplicon of 3.4kb (**Fig. 2.2**). pRN2 was confirmed by restriction digestion with *Sac1* to release 3.2kb *ptac-csYF-citC* and 2.9kb pJET (**Fig. 2.3**). pRN3 was confirmed by PCR of *tac-pqq* using primers F1 and R2 to give amplicon of 3.4kb (**Fig. 2.3**). PQQ levels found to be 5.6 ± 0.12 μg PQQ/ml of culture from both *Ec*N-21 and *Ec*N-20 transformants by growing at 37°C in M9 minimal medium after 24 h using glucose as carbon source. Both transformants dropped the pH of Tris-buffered medium with appearance of red colour due to secretion of gluconic acid by the activity of glucose dehydrogenase using PQQ as cofactor. Furthermore, citric acid levels secreted by *Ec*N-21 found to be 2 ± 0.09 mM.

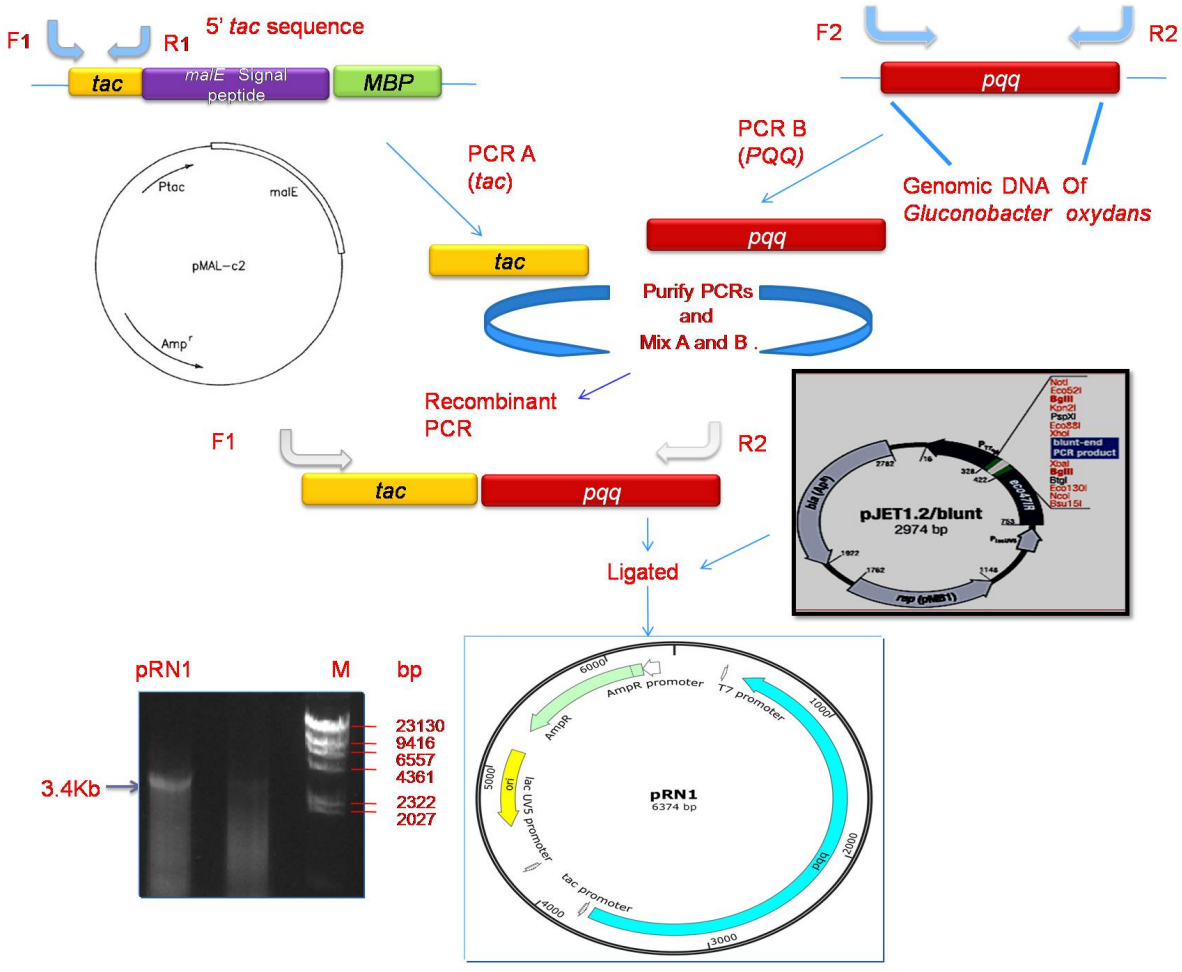

Fig. 2.2. Cloning strategy for construction of pRN1and its confirmation by PCR.

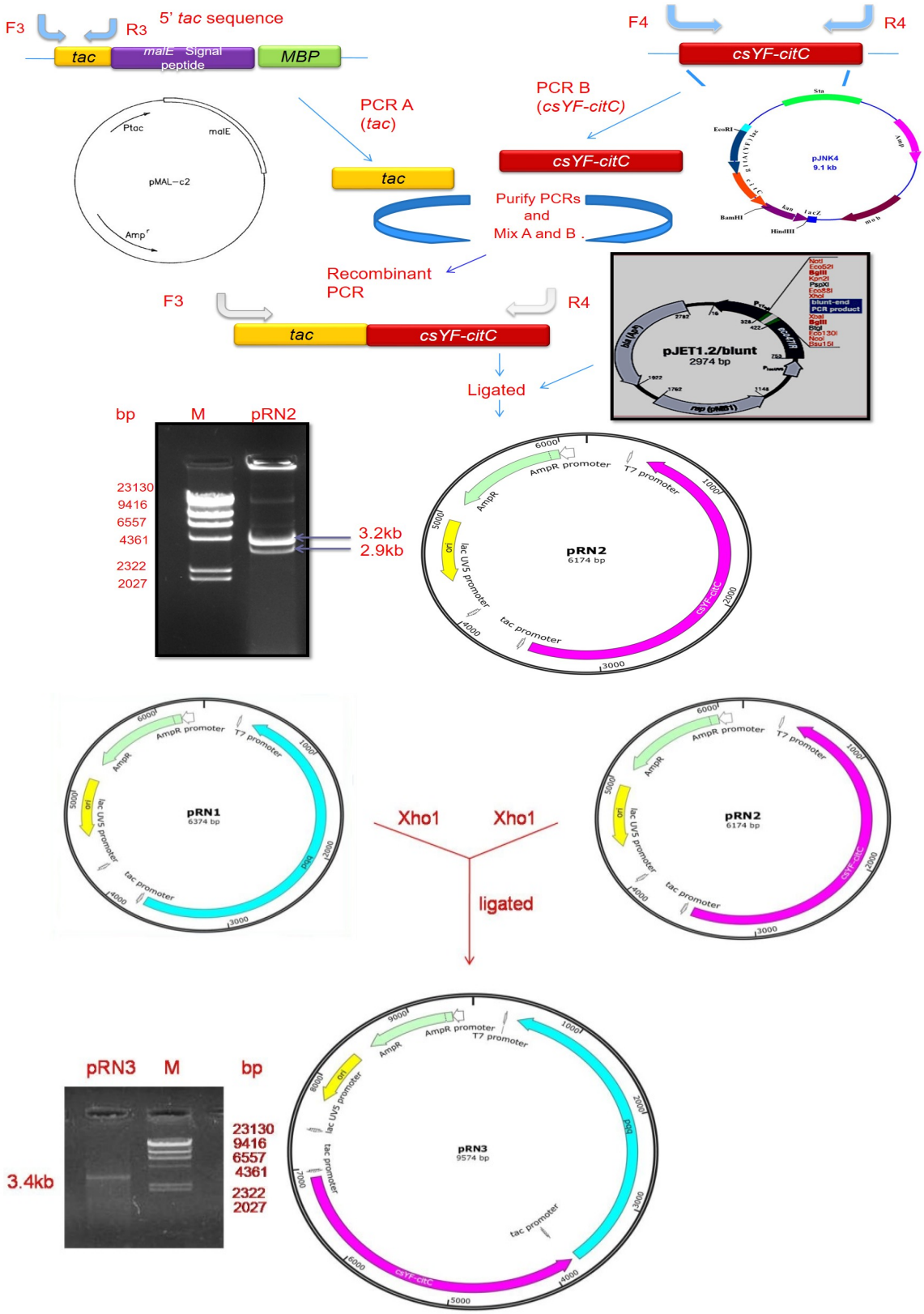

Fig. 2.3. Cloning strategy for construction of pRN2 and pRN3; the confirmation of pRN2 by restriction digestion with Sac1 and of pRN3 by PCR.

2.3.2. Effect of *Ec*N-2 transformants against Cd induced liver and kidney damage in rats.

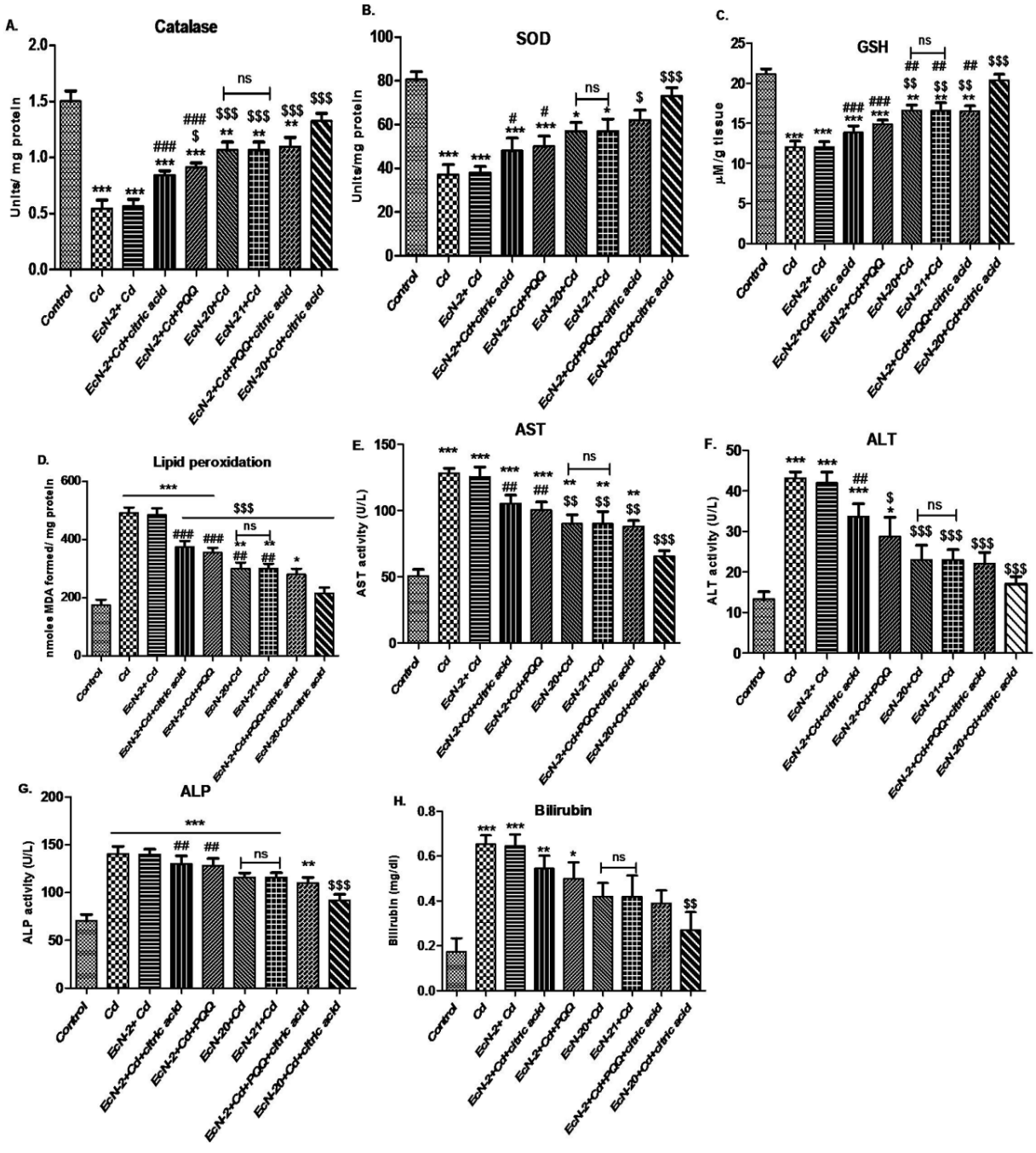

Fig. 2.4. Effect of genetically engineered probiotic *E. coli* Nissle 1917 in liver on Cd exposure: (A) Catalase, (B) SOD activity, (C) GSH levels, (D) Lipid peroxidation in liver, and (E) AST, (F) ALT, (G) ALP activity, (H) Bilirubin levels in serum. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, ** P ≤ 0.01 and ***P ≤ 0.001 compared to Control group. $^{\$}$P ≤ 0.05, $^{\$\$}$P ≤ 0.01, $^{\$\$\$}$P ≤ 0.001 compared to Cd group, $^{\#}$P ≤ 0.05, $^{\#\#}$ P ≤ 0.01 and $^{\#\#\#}$P ≤ 0.001 compared to *Ec*N-20+citric acid (oral)+Cd.

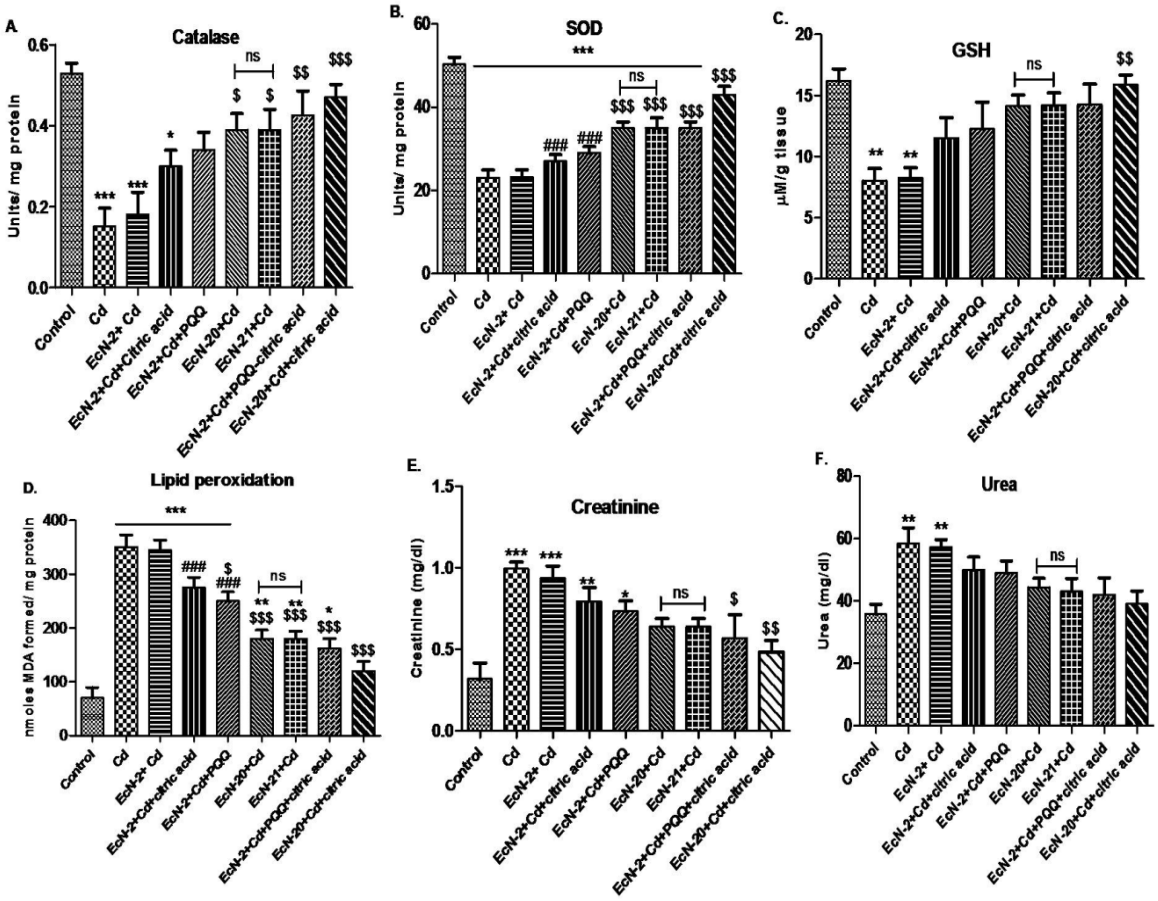

Fig. 2.5. Effect of genetically engineered probiotic *E. coli* Nissle 1917 in kidney on Cd exposure: (A) Catalase, (B) SOD activity, (C) GSH, (D) Lipid peroxidation in kidney, and (E) Creatinine, (F) Urea levels in serum. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, ** P ≤ 0.01 and ***P ≤ 0.001 compared to Control group. $P ≤ 0.05, $$P ≤ 0.01, $$$P ≤ 0.001 compared to Cd group, ###P ≤ 0.001 compared to *Ec*N-20+citric acid (oral)+Cd.

One month exposure for Cd decreased the activities of catalase and SOD as well as GSH levels while the levels of Lipid peroxidation were increased significantly in liver and kidney as compared to control (**Fig. 2.4 and Fig. 2.5**). Cd also increased the activity of hepatic damage marker enzymes such as AST, ALT and ALP as well as bilirubin, urea and creatinine levels significantly in serum compared to control whereas *Ec*N-2 treatment had no effect (Fig.1-4). However, in comparison to *Ec*N-2+citric acid (oral), *Ec*N-2+PQQ (oral), *Ec*N-20, *Ec*N-21 and *Ec*N-2+PQQ (oral)+citric acid (oral), the most effective treatment was found to be of *Ec*N-20+citric acid (oral) in increasing GSH levels, Catalase and SOD activities as well as in decreasing the Lipid peroxidation in liver and kidney, also in decreasing AST, ALT and ALP activities, bilirubin, creatinine and urea levels in

serum. *Ec*N-20 producing PQQ was more effective than *Ec*N-2+PQQ (oral) in increasing the GSH levels, Catalase and SOD activity as well as in decreasing Lipid peroxidation in liver and kidney, also in decreasing AST, ALT and ALP activities, bilirubin and creatinine levels in serum. However, protection shown by *Ec*N-21 is found to be same as that of *Ec*N-20.

2.3.3. PQQ Quantification from faeces and liver

The levels of PQQ found to be 4.45±0.2, 4.51±1.16, 4.68±0.19 nmoles/g wet wt of feces and 235.51±5.6, 224.64±8.1, 250.20±8.3 picomoles/g liver tissue in Group VI, VII and IX which are significantly higher compared to control and GroupV (**Table 2.4**).

Cd Groups	I	II	III	IV	V	VI	VII	VIII	IX
Fecal (n moles/ g fecal wet weight)	0.697±0.12	0.709±0.19	0.728±0.13	0.717±.23	0.779±0.15	4.45±0.2***	4.51±1.16***	0.764±0.23	4.68±0.19***
Liver (picomoles/ g tissue)	23.16±7.1	25.12±4.1	24.64±3.4	26.51±5.1	30.11±7.8	235.51±5.6***	224.64±8.1***	31.05±5.4	250.20±8.3***

***$P \leq 0.001$ compared to Group I and Group V

Table 2.4. PQQ Concentration in fecal matter and liver Homogenate of rats

2.3.4. Cd Estimation

Levels of Cd in faecal matter levels were significantly higher in Groups IV, VIII and IX with orally administered citric acid as compared to Cd, *Ec*N-2+Cd, *Ec*N-2+Cd+PQQ (oral), *Ec*N-20+Cd, *Ec*N-21+Cd (**Fig. 2.6**).

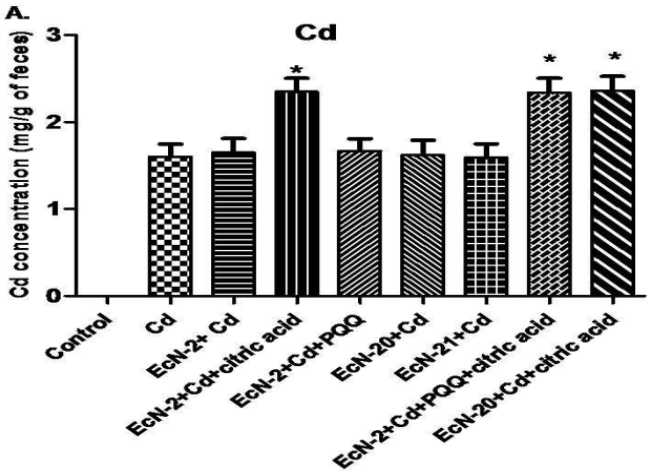

Fig. 2.6. Effect of genetically engineered probiotic *E. coli* Nissle 1917 on levels of Cd in faeces of rats. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05 compared to Cd, *Ec*N-2+Cd, *Ec*N-2+Cd+PQQ (oral), *Ec*N-20+Cd, *Ec*N-21+Cd.

2.3.5. Histological analysis

Histopathological studies showed the normal architecture in control group while Cd treatment induced pathological changes in liver and kidney (**Fig. 2.7 and 2.8**). Cd exposure caused derangement of hepatic cords, hepatocyte vacuolation, necrosis, inflammatory cell infiltration, perinuclear halo, and sinusoidal dilatation whereas in kidney leads to necrotic renal tubules, renal tubular dilatation, distention of Bowmen's cavity, shrinkage and reducing size of glomeruli. *Ec*N-20+citric acid (oral) treatment resulted in near to normal appearance in liver while in kidney moderate improvement of renal tubules was observed along with noticeable glomerulonephritis and missing mesangial space.

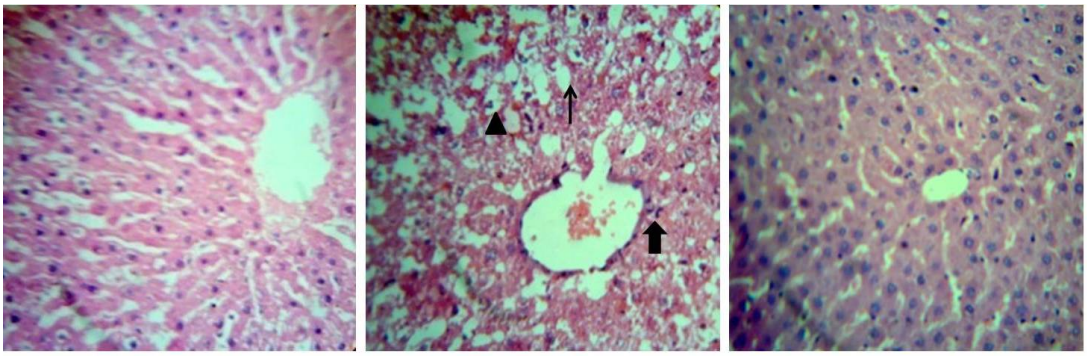

Fig. 2.7. Photomicrograph of liver stained with HE (Magnification=40x). (A) Control showing normal liver architecture, (B) Cd treated group showing Hepatocyte Vacuolation (black arrow), Perinuclear halo (arrow head), necrosis (bold arrow), (C) *Ec*N-20+Cd+citric acid (oral) showing near to normal appearance of liver .

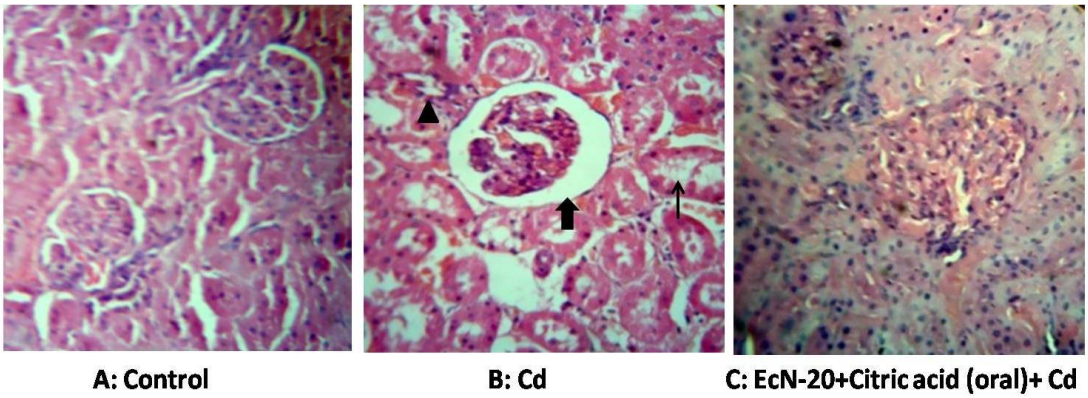

Fig. 2.8. Photomicrograph of kidney stained with HE (Magnification=40x). (A) Control showing normal kidney architecture, (B) Cd treated group showing renal tubular dilatation (black arrow), renal tubular necrosis (arrow head), shrinkage of glomeruli and distention of bowman's capsule (bold arrow), (C) *Ec*N-20+Cd+citric acid (oral) showing moderate improvement of renal tubules, with detected glomerulonephritis with missing mesangial space.

2.4. Discussion and conclusion

Contaminated food and water are the major sources of exposure of heavy metals such as Cd (Ercal et al., 2001). Damage caused by Cd in liver and kidney are mainly proportionate to the quantity of Cd present in free form not bound to Metallothionein (MT). Body burden of heavy metals can be decreased by MT due to their complex forming ability with heavy metals where excess of metal could cause saturation of Mt that leads to accumulation of unbound free metal form causing subsequent damage (Garcia-Nino and Pedraza-Chaverri, 2014). Heavy metals can cause oxidative stress by

interacting with sulfhydryl groups present in GSH and located at the active sites of antioxidant enzymes, thereby decreasing their levels (Quig et al., 1998; Hultberg et al., 2001). In addition, heavy metals can also inhibit the activity of antioxidant enzymes by replacing cofactor of these enzymes (Donaldson et al., 1991). For instance, Cd increases the levels of iron and copper by replacing them with in ceruloplasmin and ferritin, thereby generating free radicals by the Fenton reactions (Waisberg et al., 2003). Several animal experiments have suggested that in liver and kidney Cd disturbed the activities of antioxidant enzymes as well as GSH and MDA levels (Shaikh et al., 1999; Mahboob et al., 2001; Ognjanovic et al., 2003). Cd mediated increase in ROS can also cause tubular injury ultimately increasing urea and creatinine levels in blood (Necib et al., 2014). Acute liver damage characterized by increase in levels of AST, ALT, ALP and bilirubin (Naik et al, 2011; Kang et al., 2013). Similarly, the present study demonstrated a significant damage to liver and kidney due to Cd which is in agreement with the previous observations.

Gut microbiota grant several defence mechanisms against heavy metals, for instance, by sequestering heavy metals or by transforming to a less toxic form or by actively transporting metals out of the cell (Mowll et al., 1984; Hamlett et al., 1992; White et al., 1998). Probiotic *Lactobacillus plantarum* CCFM8610 treatment was found to prevent hepatic and renal oxidative stress, decrease Cd accumulation in tissues with improved hepatic tissue architecture (Zhai et al., 2013). In current study, *Ec*N-20 was found to be more effective than *Ec*N-2 with PQQ (oral) while *Ec*N-2 was not effective. Likewise, similar results were shown by probiotic *E. coli* CFR 16 and *Ec*N secreting PQQ against damage occured on DMH, alcohol and rotenone treatment (Pandey et al., 2014; Singh et al., 2014; Singh et al., 2015). However, the most effective treatment was *Ec*N-20 with citric acid (oral).

EDTA, DMPS, DMSA and BAL are the most widely used clinically important chelators (Bernhoft et al., 2013). Perhaps, chelators mobilize heavy metals from kidney for excretion in urine and from liver through bile (Rooney et al., 2007). Protective mechanism behind the function of *Ec*N-20+citric acid (oral) is perhaps due to constitutive

production of PQQ within gut by *Ec*N-20, wherein PQQ get absorbed by enterocytes and then reaches to different organs like liver and kidney where it scavenges ROS produced

by Cd. Along with administration of citric acid will chelate excess Cd in liver and kidney (Kojima et al., 1976). Both *Ec*N-20 and *Ec*N-21 produce PQQ which acts as a cofactor for glucose dehydrogenase which facilitates the conversion of glucose to gluconic acid. Gluconic acid is known to acts as prebiotic (Kameue et al., 2004) and utilized by beneficial microbiota results into production of SCFAs (Short chain fatty acids) which is reported to provide anti-inflammatory effects (Cox et al, 2009) and could also prevent against pro-inflammatory effects of Cd. This is supported from our previous study where the feacal matter from the rats treated with *Ec*N secreting PQQ had high levels of SCFAs such as butyric and propionic acids (Singh et al, 2014). According to histopathological studies, the present strategy is more effective for liver as compared to kidney as it is the major target organ affected by heavy metal toxicity (Yang et al., 2015). *Ec*N-21 producing PQQ and citric acid was found to be equally effective as *Ec*N-20 producing PQQ alone due to the lower levels of citric acid produced by *Ec*N-21 which leads to incompetent chelation of Cd. Therefore, proposed strategy in this work has moderate protective effect on Cd toxicity and paves the way for improvising the probiotics for better protection by increasing the levels of chelators.

To conclude, present study demonstrates that *Ec*N-20 producing PQQ supplemented with citric acid orally is an effective strategy against Cd induced liver and kidney damage as compared to orally given PQQ and citric acid. However, this strategy appears to be not as effective in preventing kidney damage. Additionally, *Ec*N-21 is less effective which could be attributed to lower levels of secreted citric acid.