Chapter 5

Evaluating the efficacy of probiotic *Escherichia coli* Nissle 1917 strain containing gluconate dehydrogenase (*gad)* and pyrroloquinoline quinone (*pqq)* gene cluster against long term coexposure of cadmium and lead in rats.

5.1. Introduction

In chapters 3 and 4, *Ec*N-23 producing PQQ and 2-ketogluconic acid was shown to be effective against Cd induced toxicity as well as for immunotoxicity of Pb in LPS/GalN treated rats. Nevertheless, all these experiments were conducted for a period of one month only and coexposure studies are yet to be performed. Coexposure of Cd and Pb can generate reactive oxygen species leading to liver and kidney damage, it can also induce apoptosis henceforth impairing their function. Additive effect of Cd and Pb is neurotoxic too by disabling blood brain barrier (Matovic et al. 2015; Yuan et al. 2014a; Yuan et al. 2014b; Tobwala et al 2014). Dislipidemia can also be caused by Cd and Pb which could be overcome by using quercetin and ascorbate/DMSA respectively (Prabhu et al., 2013; Ugbaja et al., 2013). Therefore, the present strategy was designed to evaluate the effect of *Ec*N-23 producing PQQ and 2-ketogluconic acid against long term (4 months) coexposure of Cd and Pb.

5.2. Methods and materials

5.2.1. 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).**

5.2.2. Bacterial strains and culture conditions

Probiotic strains including *Ec*N-2, *Ec*N-22, *Ec*N-23 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.

5.2.3. Experimental design

To determine the effect of *Ec*N-22 and *Ec*N-23 on long term coexposure of Cd and Pb, rats were divided into 5 groups (6 rats in each group): Control, Cd-Pb, Cd-Pb+*Ec*N-2, Cd-Pb+*Ec*N-22, and Cd-Pb+*Ec*N-23. Probiotic treatment was given for 3 consecutive days following streptomycin wash, thereafter, colonization was confirmed after 7 days of treatment by fecal count. Afterwards, 100 ppm Cd and 100 ppm Pb were given together in drinking water for 4 months followed by further probiotic treatments which were given once a week for 4 months.

5.2.4. Preparation of tissue homogenates

Similar as described in section 2.2.6 of chapter 2.

5.2.5. Biochemical assays

Catalase activity was determined by protocol of Beers and Sizer (1952). Superoxide dismutase activity was detected according to method described in Marklund and Marklund (1974). Reduced GSH was determined by the method of Beutler et al. (1969). Lipid peroxidation was measured by estimating the levels of MDA according to the method described by Buege and Aust (1978). ALAD activity was measured by protocol of Berlin & Schaller (1974) , ROS estimation was done by method of Socci et al. (1999). Blood free fatty acids (FFAs) were determined by the method of Lauwerys (1969).

5.2.6. ALT, AST, ALP, urea, creatinine, bilirubin, Ca, Mg, Zn, Fe levels and Blood lipid estimaton

ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), urea, creatinine, bilirubin, Ca (Calcium), Mg (Magnesium), Zn (Zinc) and Fe (Iron) levels as well as triglycerides, HDL cholesterol, total cholesterol in serum were measured using kit as per manufacturer protocol (Beacon Diagnostics Pvt. Ltd., Navsari, Gujarat, India).

5.2.7. Histopathological changes

Similar as described in section 2.2.9 of chapter 2.

5.2.8. mRNA expression and quantitative reverse transcription PCR

From liver RNA was extracted with Trizol (Invitrogen BioServices India Pvt. Ltd., Bangalore, India), thereafter, cDNAs were constructed using 1μg total RNA following the manufacturer's instructions (Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Primers used for Metallohtionein-II were GCAAGAAAAGCTGCTGTT (forward) and GTGTGGAGAACCGGTCA (reverse) and ABI Quant-StudioTM 12K flex Real Time PCR system coupled with SYBR Green technology (Applied Biosystems) was used for amplification. The software provided with the thermocycler (QuantStudioTM) was used to analyze the linearity of the dissociation curve. Samples in duplicates were analyzed.

5.2.9. Metal determination

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

5.2.10. Statistical analysis

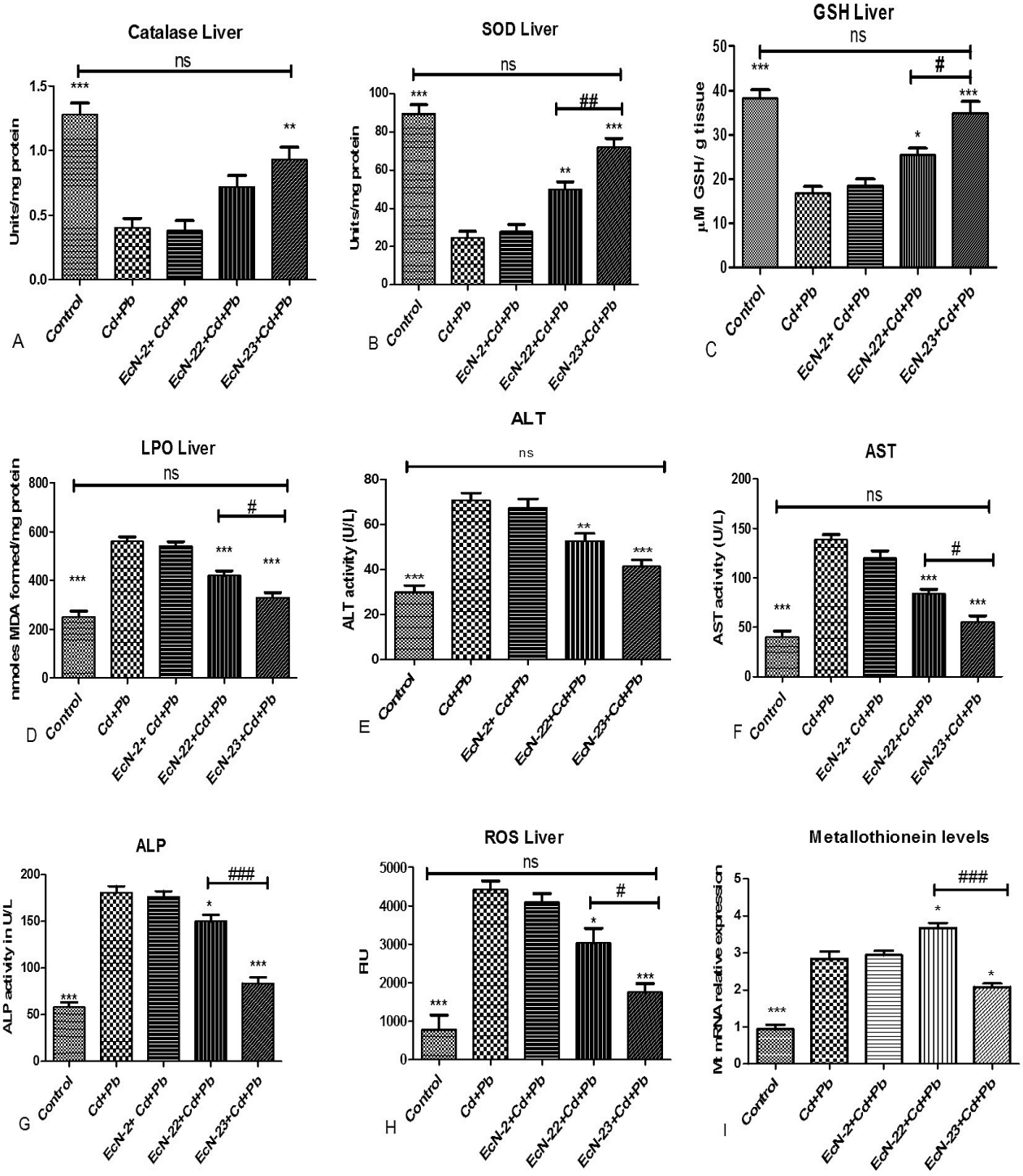
Similar as described in section 2.2.11 of chapter 2.

5.3. Results

5.3.1. Effect of *Ec*N-23 against long term coexposure of Cd and Pb in rats.

Four months coexposure of Cd and Pb decreased the ALAD activity, body weight, haemoglobin levels, the Catalase and SOD activities as well as GSH levels while the lipid peroxidation was significantly increased in liver and kidney; but ROS and Mt levels were significantly increased in liver whereas in serum urea, creatinine, bilirubin levels as well as AST, ALT, ALP activities were also significantly increased compared to control (**Fig. 5.1; Table 5.3**). *Ec*N-23 was more effective as compared to *Ec*N-22 while *Ec*N-2 was not effective in restoring the ALAD activity, body weight, haemoglobin, Catalase and SOD activities, GSH levels, Lipid peroxidation in liver and kidney; ROS and Mt levels in liver as well as AST, ALT, ALP activities and urea, creatinine, bilirubin levels in serum.

In serum the levels of essential metals like Calcium, Zinc, Iron and Magnesium were significantly decreased by Cd and Pb coexposure as compared to control without any significant loss of these essential metals was observed on *Ec*N-23 treatment, nevertheless, their absorption was enhanced by *Ec*N-23 by enhancing the loss of Cd and Pb (**Fig. 5.2**). In Cd-Pb treated group SCFAs were significantly decreased while their levels increased to near normal on synbiotic treatment of *Ec*N-22 and *Ec*N-23 (**Table 5.1**). In liver and faecal matter, PQQ levels were significantly high in *Ec*N-22 and *Ec*N-23 treated groups, detectable levels of 2-KG were found in serum and faecal matter in *Ec*N-23 treated group (**Table 5.2**). Furthermore, in *Ec*N-23 treated group, the release of Cd and Pb in faeces was enhanced as determined by Atomic absorption spectroscopy (**Table 5.2**). In Cd-Pb treated group serum total cholesterol (TC), triglycerides (TG), free fatty acid levels were increased and HDL-cholesterol was decreased significantly while *Ec*N-23 treatment had brought the levels near to control (**Table 5.3**).


Catalase Liver
Units/mg protein
SOD Liver
Units/mg protein
GSH Liver
µM GSH/ g tissue
LPO Liver
nmoles MDA formed/mg protein
ALT
ALT activity (U/L)
AST
AST activity (U/L)
ALP
ALP activity in U/L
ROS Liver
RFU
Metallothionein levels
Mt mRNA relative expression
ns
Control
Cd+Pb
EcN-2+ Cd+Pb
EcN-22+Cd+Pb
EcN-23+Cd+Pb
A
B
C
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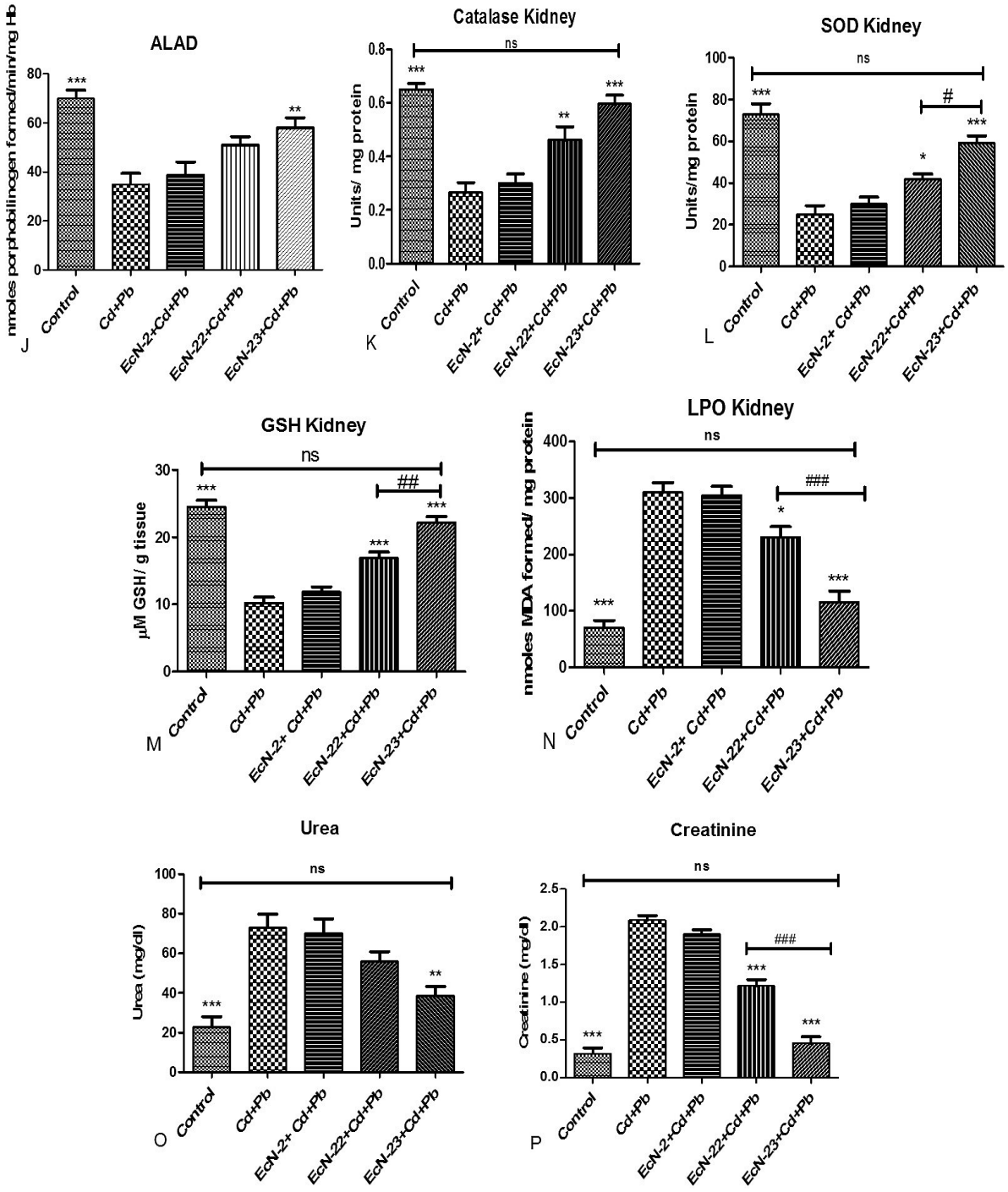

Fig. 5.1. Effect of genetically engineered probiotic *E. coli* Nissle 1917 on Cd-Pb coexposure: (A) Catalase, (B) SOD activity, (C) GSH levels, (D) Lipid peroxidation in liver, (E) ALT, (F) AST, (G) ALP activity in serum, (H) ROS levels in liver, (I) mRNA expression levels of Metallothionein in liver, (J) ALAD activity in blood, and in kidney (K) Catalase, (L) SOD activity, (M) GSH levels, (N) Lipid peroxidation, (O) Urea, (P) Creatinine levels in serum. Values are expressed as mean ± SEM (n=6 each group). * P ≤ 0.05, ** P ≤ 0.01,*P ≤ 0.001 compared to Cd-Pb. $^{\#}$P ≤ 0.01, $^{\#\#}$P ≤ 0.05 $^{\#\#\#}$P ≤ 0.001 compared to *Ec*N-22+Cd+Pb group.**

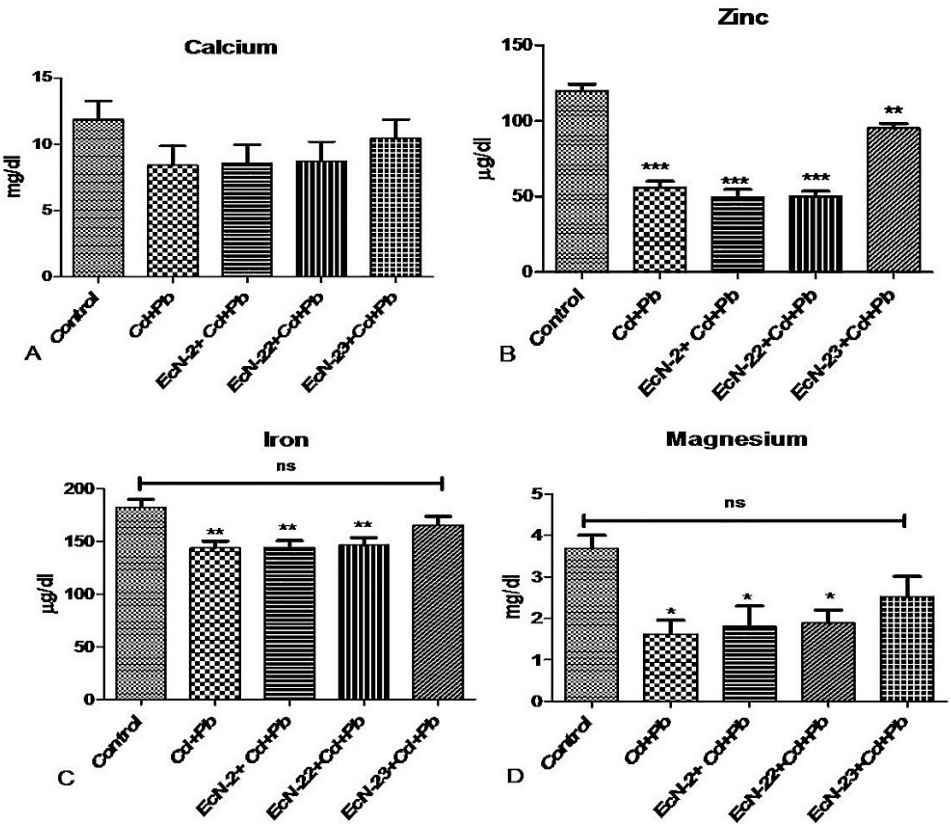

Fig. 5.2. Effect of genetically engineered probiotic *E. coli* Nissle 1917 on: (A) Calcium, (B) Zinc, (C) Iron, and (D) Magnesium levels in serum. Values are expressed as mean ± SEM (n=6 each group).* P ≤ 0.05, ** P ≤ 0.01,*P ≤ 0.001 compared to Control.**

Groups	Control	Cd-Pb	*Ec*N-2	*Ec*N-22	*Ec*N-23
Acetate	**70.23±2.4**	**62.44±3.1**	**64.75 ±2.8**	**81.42 ±2.6a* b*****	**90.14±3.7a***b*****
Propionate	**20.41±1.3**	**13.42±1.7a***	**15.18±1.4**	**21.29±1.6b***	**28.31±1.5a*b***c***
Butyrate	**9.9±0.7**	**5.4±0.9a***	**5.9±1.2**	**14±1.3b*****	**19±0.8a***b***c***

a*$p \leq 0.05$, a*$p \leq 0.001$** compared to Control. **b*$p \leq 0.05$, b***$p \leq 0.001$** compared to Cd-Pb**. c*$p \leq 0.05$** compared to *Ec*N-22. Values are expressed as μmoles/g colonic content. Values are mean±SEM (6 rats each group).

Table 5.1. Short chain fatty acids (SCFAs) concentration in colonic matter of Cd-Pb exposed rats.

Groups	Control	Cd-Pb	*Ec*N-2	*Ec*N-22	*Ec*N-23
PQQ					
Fecal (n moles/ g fecal wet weight)	**0.602±0.11**	**0.641±0.14**	**0.676±0.19**	**1.6 ±.13*** **	**1.8 ±0.12 *** **
Liver (picomoles/ g tissue)	**26.34±6.4**	**24.54±8.1**	**28.33±5.8**	**108.51±4.9*** **	**102.98±6.3*** **
2-KG					
Fecal (*μ*moles/ g fecal wet weight)	**nd**	**nd**	**nd**	**nd**	**200±7.9**
Serum (ng/ml)	**nd**	**nd**	**nd**	**nd**	**0.72±0.8**
***$p \leq 0.001$** compared to Control					
Cd (mg/g feces)	**nd**	**2.1±0.12**	**2.3±0.17**	**2.5±0.14**	**3.8±0.19 *** **
Pb (mg/g feces)	**nd**	**1.4±0.11**	**1.7±0.13**	**1.8±0.15**	**2.9±0.18*** **
***$p \leq 0.001$** compared to Cd-Pb, *Ec*N-2, *Ec*N-22					

Table 5.2. PQQ and 2-Ketogluconic acid (2-KG) concentration in fecal matter, liver homogenate and serum of rats; Cd and Pb levels in feces.

Groups	Control	Cd-Pb	*Ec*N-2	*Ec*N-22	*Ec*N-23
Total cholestrol (mg/dl)	**58.21±2.31 a*** **	**83.42±2.87**	**80.17±3.37**	**73.33±2.51**	**66.58±2.64 a** **
Triglycerides (mg/dl)	**45.47±3.14a*** **	**77.43±1.69**	**73.69±1.83**	**65.52±2.72a***	**58.24±3.11a*** **
HDL (mg/dl)	**40.41±1.46 a*** **	**28.52±1.39**	**29.49±2.04**	**33.61±1.99**	**36.98±1.47a***
Free fatty acids (mg/dl)	**15.51±3.32a***	**32.21±2.92**	**30.83±3.12**	**26.38±3.82**	**21.61±2.84**
Body weight change (g)	**180±7.74a*** **	**90±6.87**	**95±8.37**	**140±7.51a** **	**160±7.64 a*** **
Hb (g/dl)	**15.51±0.32a*** **	**10.11±0.22**	**10.91±0.29**	**12.14±0.31a*** **	**14.33±0.19a*** b*** **
Bilirubin (mg/dl)	**0.33±0.048a*** **	**1.06±0.064**	**0.87±0.036**	**0.60±0.057a** **	**0.45±0.068a*** **

a*$p \leq 0.05$, a$p \leq 0.01$**, a***$p \leq 0.001$** compared to Cd-Pb, **b***$p \leq 0.001$** compared to *Ec*N-22

Table 5.3. Serum lipid profile, body weight change, haemoglobin and bilirubin levels in rats.

5.3.2. Histopathological changes

In control the normal architecture of liver and kidney was observed after histological analysis while drastic histopathological changes were observed on Cd+Pb treatment (**Fig. 5.3**). Exposure of Cd+Pb caused hepatocyte vacuolation, inflammatory cell infiltration and necrosis, derangement of hepatic cords, perinuclear halo while in kidney, it leads to inflammatory cell infiltration and renal tubular necrosis, glomerulonephritis with missing

mesangial space, renal tubular dilatation. Significant recovery of histopathological damage was observed in liver and kidney on *Ec*N-23 treatment.

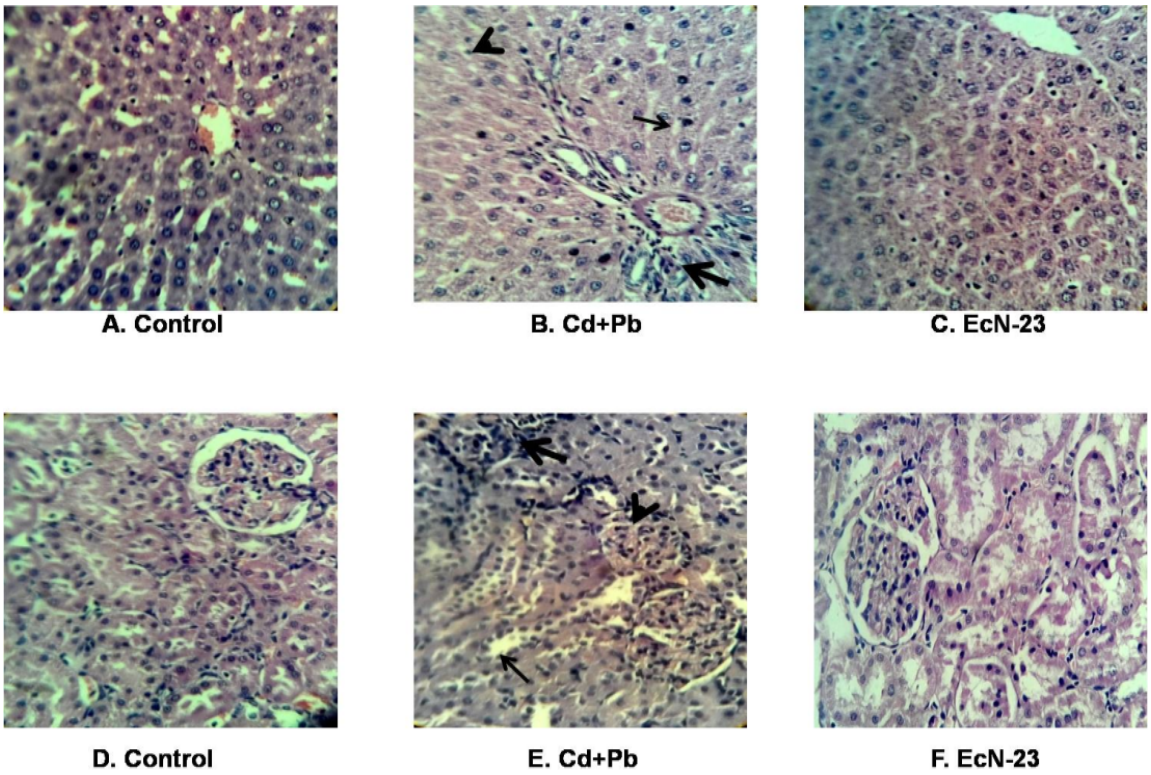

Fig. 5.3. Photomicrograph of liver stained with HE (Magnification=40x). (A) Control showing normal liver architecture, (B) Cd-Pb treated group showing inflammatory cell infiltration and necrosis (bold arrow), hepatocyte Vacuolation (black arrow), Perinuclear halo (arrow head), derangement of hepatic cords, (C) *Ec*N-23+Cd+Pb showing near to normal appearance of tissue architecture and in kidney (A) Control showing normal kidney architecture, (B) Cd-Pb treated group showing inflammatory cell infiltration and renal tubular necrosis (bold arrow), renal tubular dilatation (black arrow), glomerulonephritis with missing mesangial space (arrow head), (C) *Ec*N-23+Cd+Pb showing near to normal appearance of tissue architecture.

5.4. Discussion and conclusion

Combination of Cd and Pb can show additive or synergistic interactions or other effects not observed by either of single metal treatment (Wang and Fowler, 2008). Their mechanism of toxicity mainly involves inhibition of sulfhydryl group containing enzymes and induction of ROS production (Navas-Acien et al., 2009). In rats with coexposure to Pb and Cd exacerbate cytotoxicity in proximal tubular cells (Wang et al., 2011). Oral administration of low to moderately high levels of Cd and Pb to mice showed increased Cd

levels in kidney as compared with Cd in kidney of mice fed on Cd (Exon et al., 1978). The combination had also caused marked reduction in haemoglobin or hematocrit levels (Thawley et al., 1977). Urinary ALA was observed to be moderate for the mixture of Cd and Pb, however, it was found to be increased for Pb alone but it is not the case for Cd. The Cd and Pb coexposure has additive effect on decreasing the body weight (Buchet et al., 1981). Likewise, results obtained in the present study are concomitant with previous studies where significant increase in oxidative damage had been reported with marked increase in ROS levels leading to liver and kidney damage on Cd and Pb coexposure as characterized by respective damage markers.

Increase in metal mobilization with improved biochemical variables were observed with supplementation of antioxidant along with chelating agents (Flora, 2002; Pande and Flora, 2002). Renal and hepatic oxidative stress induced by Cd was found to be reduced in response to application of *Lactobacillus plantarum* CCFM8610 by reducing the accumulation of Cd within tissues which is correlated with improved hepatic histopathology (Zhai et al., 2013).

The use of chelating agents also involves the serious side effects including the disturbed homeostasis of essential metal ions along with complexed metal ions dislocation to the dangerous body sites (Crisponi et al., 2015). Therefore, to ensure the safety of *Ec*N-23, four months long term experiments were designed. The reliability for longer exposure of *Ec*N-23 treatment was confirmed by decrease in hepatotoxicity and nephrotoxicity associated with coexposure of Cd and Pb. *Ec*N-2 was found to be ineffective in dealing with toxicity associated with Cd and Pb due to inability of *vgb* integrated in genome to restore the damage, however, *Ec*N-22 was found to be more effective than *Ec*N-2. Similar results were shown by Probiotic *E. coli* CFR 16::v*gb-gfp* (*pqq*) and *Ec*N secreting PQQ against oxidative stress induced by administration of DMH, alcohol and rotenone (Pandey et al. 2014; Pandey et al. 2015; Singh et al. 2014; Singh et al. 2015).

Competitive inhibition by Cd and Pb for the uptake of Ca, Mg, Zn and Fe to intestinal metal uptake transporter like DMT1 (divalent metal transporter-1) and MTP1 (metal transporter protein-1) prevents their absorption (Zhai et al., 2015). In the present study, in Cd and Pb treated groups the levels of Ca, Mg, Fe and Zn were decreased. Significant loss

of calcium and zinc along with Pb and Cd was seen on EDTA chelation therapy (Flora and Pachauri, 2010). However, no significant decrease in plasma levels of calcium, magnesium, zinc and iron was seen on application of N-acetylcysteine which acts as both antioxidant and heavy metal chelator (Waters et al., 2001; Hjortso et al., 1990). Similarly, levels of Ca, Mg, Fe and Zn were unaltered in the present study after *Ec*N-23 treatment with actual increase in levels of essential metals due to the chelation of Pb and Cd shown by 2-ketogluconic acid, thus preventing competitive inhibition of transporters and leading to increase in essential metal levels. This could be attributed to the continuous secretion of 2-ketogluconic acid in GI tract by *Ec*N-23 which is sufficient for complexing Pb and Cd and prevents their absorption while the levels of this acid in serum is low as shown in **Table 5.2**.

The availability and absorption of nutrients is decreased by Cd, hence body weight gain was found to be low in Cd-Pb treated group (Elsenhans et al., 1999; Eriyamremu et al., 2005) Cd and Pb exposure causes dyslipidemia by alteration in TC, TG, HDL-C and LDL-C metabolism. Cd increases the activity of HMG-CoA (hydroxy-3-methylglutaryl-coenzyme A reductase) and decreases in LDL receptor gene expression resulting in increasing the serum concentration of cholesterol (Prabu et al., 2013; Ugbaja et al., 2013). The increase in serum free fatty acids was observed due to the inhibition of β oxidation via Cd. After Cd treatment, lower level of plasma HDL-C was observed due to altered metabolism of the major HDL apoprotein. Moreover, impaired catabolism of TG-rich particles in Cd toxicity leads to increase in serum triglycerides (Prabu et al., 2013; Ugbaja et al., 2013).

Diet deficient in PQQ leads to hyperlipidemia and upregulation of lipid biosynthesis enzymes in mice by interacting with signaling pathways involved in lipid metabolism (Bauerly et al., 2011). In rats the colonic SCFAs (mainly butyrate) levels were enhanced by gluconic acid (Kameue et al. 2004). Lipid metabolism also influenced by SCFAs such as upregulation of lipid oxidizing enzymes by acetate in liver (Kondo et al. 2009), decrease in fatty acid levels in liver and plasma is mediated by propionate (Al-Lahham et al. 2010) and stimulation of ß-oxidation of fatty acids, regulation of cholesterol synthesis, proliferation of peroxisome occurs by butyrate (Canani et al. 2011). Combined effect

results in the normalized lipid profile in groups received synbiotic treatment with *Ec*N-22 and *Ec*N-23. In conclusion, *Ec*N-23 is also effective for long term coexposure of Cd+Pb in liver and kidney damage as well as against dislipidemia without affecting the essential metal ions homeostasis illustrating its safety and usefulness for therapeutic purposes.