Chapter 4

Evaluating the efficacy of probiotic *Escherichia coli* Nissle 1917 strain containing gluconate dehydrogenase (*gad)* and pyrroloquinoline quinone (*pqq)* gene cluster in amelioration of LPS/GalN induced damage in lead treated rats.

4.1. Introduction

In chapter 3, *Ec*N-23 producing PQQ and 2-ketogluconic acid was found to be an effective strategy against Cd induced liver and kidney damage. However, the effect of *Ec*N-23 on other heavy metals required to be explored. Lead (Pb) is one of the intensively studied heavy metal for immunotoxicity (Maria et al., 2000; Flohe et al., 2002; Brown et al., 1998). Exposure to Pb increases susceptibility to infections by inducing shift from Th1 mediated immune response to Th2 and eventually causing altered cytokines release. If animals are pre-exposed to Pb then they are more sensitive to LPS mediated septic shock involving series of events such as elevation in iNOS activity, neutrophil infiltration, mast cell degranulation and lipid peroxidation. Hence, the present strategy was designed to evaluate the effect of *Ec*N-23 producing PQQ and 2-ketogluconic acid against Pb induced immunotoxicity in LPS (*E. coli* O55:B5)/GalN (Galactosamine) treated rats.

4.2. Methods and materials

4.2.1. 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 (12h light: 12h 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).**

4.2.2. 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.

4.2.3. Experimental design

Rats were divided into 7 different groups (6 rats per group) to evaluate the effect of *Ec*N-22 and *Ec*N-23 on immunotoxicity of Pb in rats injected with LPS/GalN: Water, Pb, Water+LPS/GalN, Pb+LPS/GalN, Pb+LPS/GalN+*Ec*N-2, Pb+LPS/GalN+*Ec*N-22, Pb+LPS/GalN+*Ec*N-23. Following streptomycin wash, probiotic treatment was given for 3 consecutive days, thereafter, colonization of *gfp* tagged probiotics was confirmed after 7 days of treatment by fecal count. Afterwards, 500 ppm Pb was given to rats in drinking water for 4 weeks, followed by further probiotic treatments which were given once a week for 4 consecutive weeks. After 4 weeks of treatment of Pb and probiotics, intraperitoneally the rats were injected with 40 μg/kg LPS (*Escherichia coli* O55:B5) and 360 mg/kg GalN (Galactosamine) in phosphate-buffered saline in accordance with method suggested in Galanos et al. (1979). The control rats were injected with saline only. All rats were sacrificed 6 hours following the injection and then all experimental measurements were conducted. For survival experiments, 10 rats were kept in each group and mortality was observed till 24 hrs after LPS/GalN injection.

4.2.4. Preparation of tissue homogenates

Tissues were washed with ice-cold saline after sacrificing rats followed by homogenized in ice cold Phosphate Buffer Saline.

4.2.5. Biochemical assays

Lipid peroxidation was measured according to the method described by Buege and Aust (1978). Histamine was measured by the protocol of Patange et al. (2005), Gastric empting and intestinal transit was determined by the method of Souza et al. (2009), ALAD activity was measured by protocol of Berlin and Schaller (1974). NO levels were determined by the method of Green et al. (1982), Myeloperoxidase (MPO) activity was measured as described by Krawisz et al. (1984). ROS estimation was done by the method of Socci et al. (1999).

4.2.6. Histopathological changes

Similar as described in section 2.2.9 of chapter 2.

4.2.7. mRNA expression and quantitative reverse transcription PCR

From liver and colon RNA was extracted with Trizol (Invitrogen BioServices India Pvt. Ltd., Bangalore, India), thereafter, cDNA was constructed using 1μg total RNA following the manufacturer's instructions (Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Primers used for iNOS were CAACCTGCAGGTCTTCGATG (forward) and CGATGCACAACTGGGTGAAC (reverse); Metallohtionein-II were GCAAGAAAAGCTGCTGTT (forward) and GTGTGGAGAACCGGTCA (reverse); TNF-α were CCCAGAAAAGCAAGCAACCA (forward) and TG GTG GTT TGC TAC GAC GTG (reverse). 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.

4.2.8. Statistical analysis

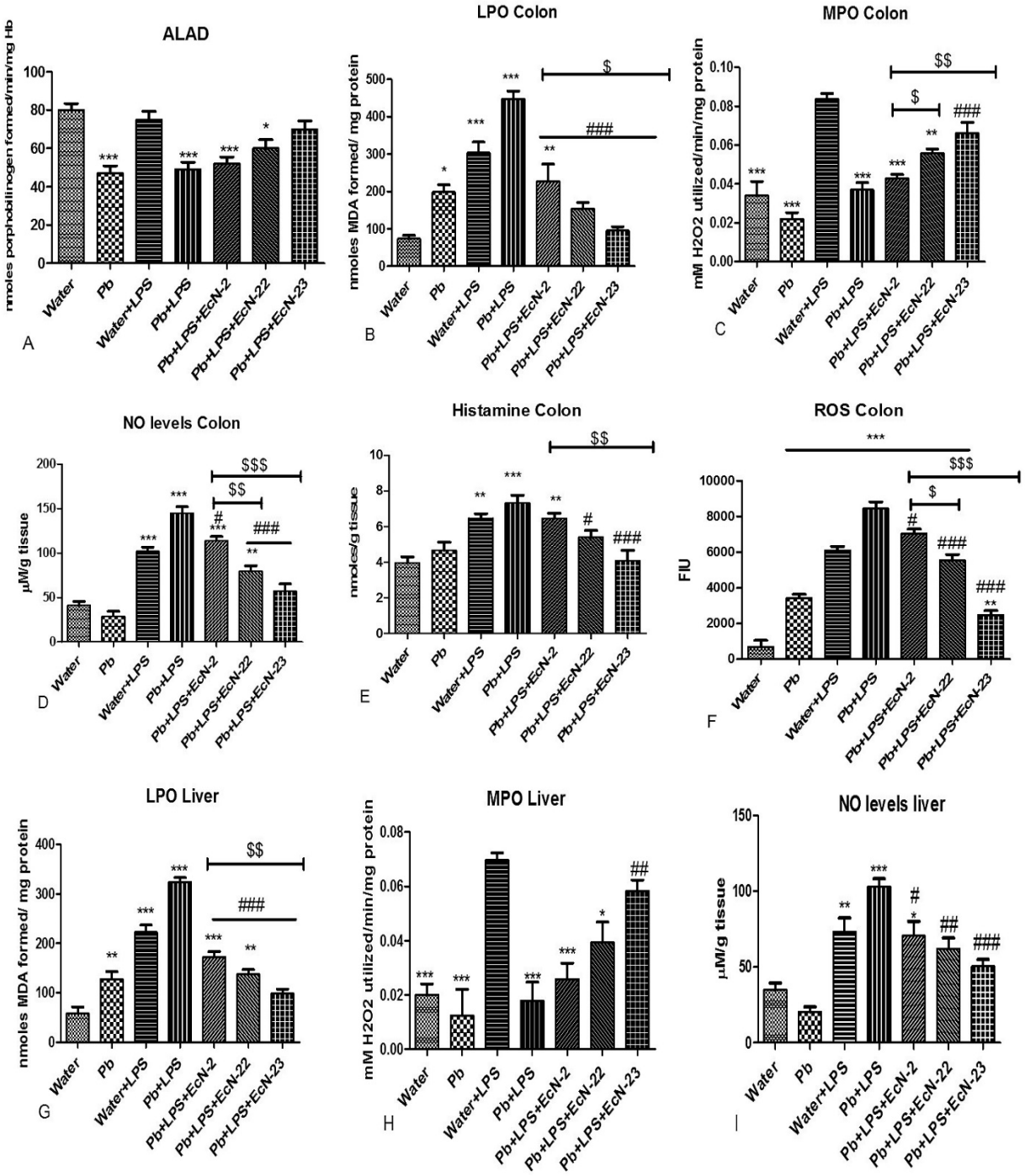
Similar as described in section 2.2.11 of chapter 2.

4.3. Results

4.3.1. Effect of *Ec*N-23 against LPS/GalN induced immunotoxicity in Pb treated rats

In blood ALAD activity was significantly decreased in Pb treated groups. Pb + LPS/GalN treatment in colon and liver significantly increased the LPO, ROS, NO and histamine levels significantly as compared to group given water only (**Fig. 4.1**). Similarly, after Pb+LPS/GalN treatment MPO activity was significantly decreased as compared to water+LPS group. In comparison with *Ec*N-2 and *Ec*N-22 treatment, *Ec*N-

23 was found to be most effective in restoring the LPO, ROS, NO, histamine levels and MPO activity.



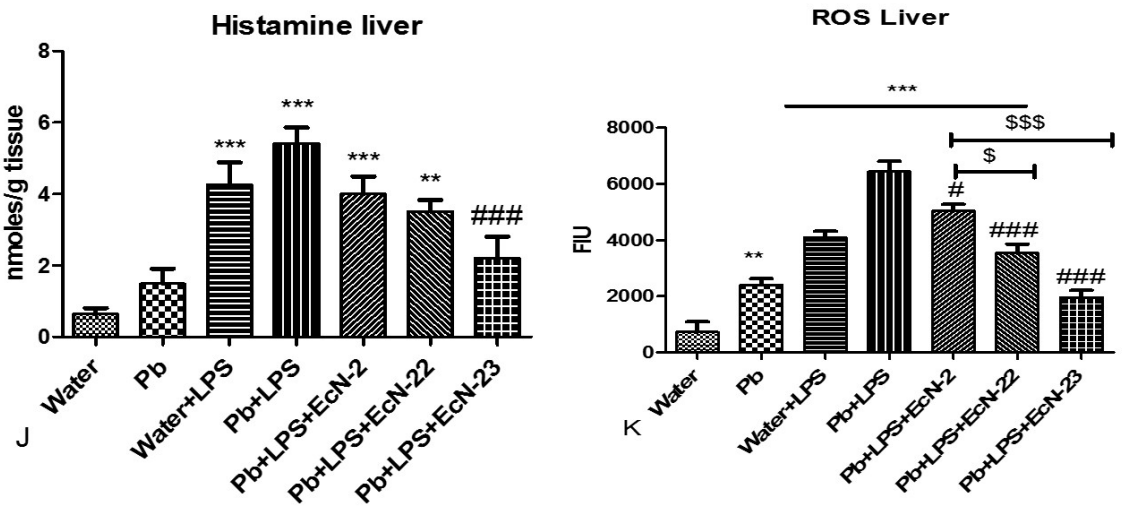


Fig. 4.1. Effect of genetically engineered probiotic *E. coli* Nissle 1917 against LPS induced damage in Pb treated rats on: (A) ALAD activity in blood, (B) Lipid peroxidation, (C) MPO activity, (D) Nitric oxide (NO) levels, (E) Histamine levels, (F) Reactive oxygen s pecies (ROS) levels in colon; and on (G) Lipid peroxidation (H) MPO activity (I) Nitric oxide (NO) levels (J) Histamine levels (K) Reactive oxygen species (ROS) levels in Liver. Values are expressed as mean ± SEM (n=6 each group). * P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001 compared to Water group. $^{\#}$P ≤ 0.05, $^{\#\#}$P ≤ 0.01, $^{\#\#\#}$P ≤ 0.001 compared to Pb+LPS group, $^{\$}$P ≤ 0.05, $^{\$\$}$P ≤ 0.01 , $^{\$\$\$}$P ≤ 0.001 compared to Pb+LPS+*Ec*N-2.

In liver mRNA levels of metallothionein (Mt) were significantly increased in groups treated with Pb as well as with LPS/GalN as compared to control (water) group, however, the maximum levels were found in group treated with Pb and LPS/GalN together (**Fig. 4.2**). Mt levels were significantly decreased after *Ec*N-23 treatment compared to Pb+LPS indicating the metal scavenging by 2-ketogluconic acid produced by *Ec*N-23. Mt levels were also observed to be high in group treated with *Ec*N-22 as compared to control (water) showing involvement of PQQ in increasing the Mt levels. mRNA levels of TNF-α and iNOS were also significantly decreased after *Ec*N-23 treatment as compared to Pb+LPS/GalN treated group in colonic tissue.

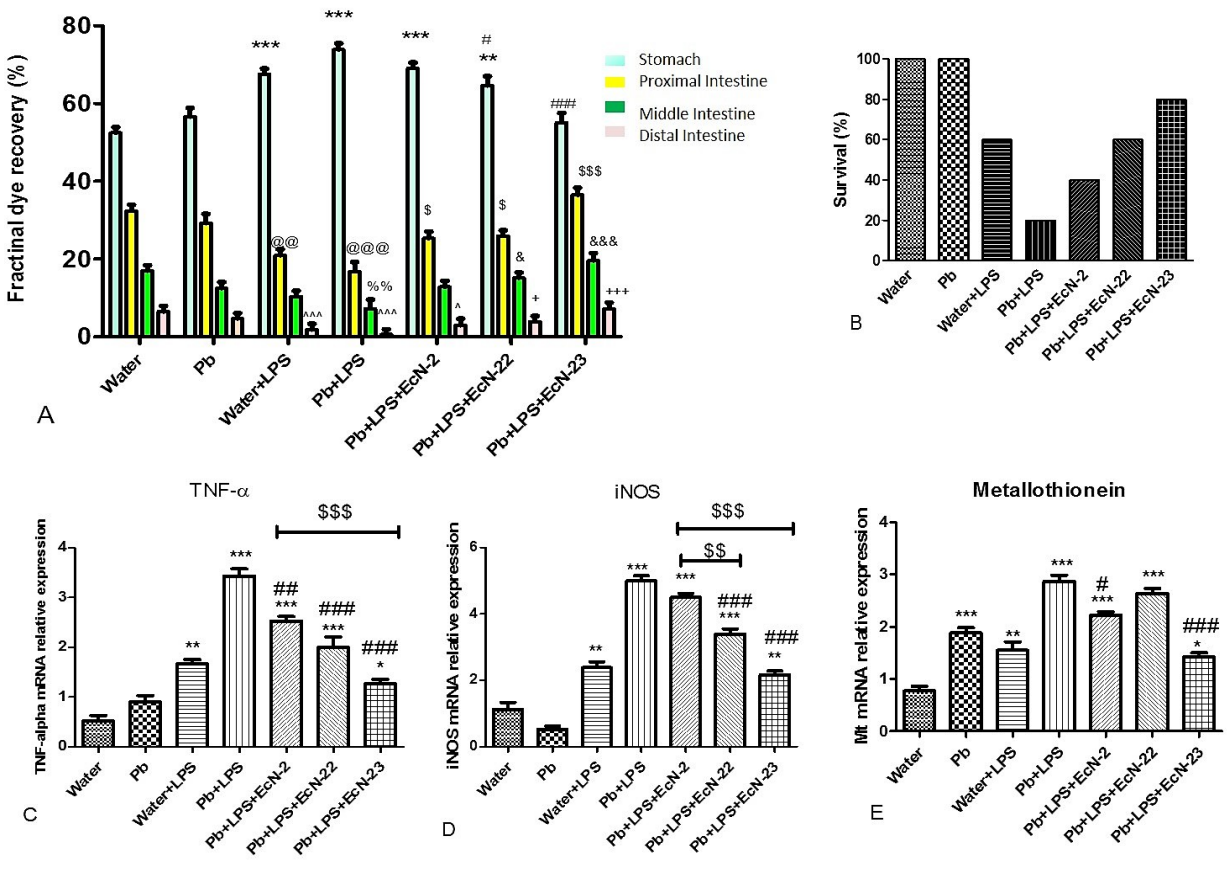


Fig. 4.2. Effect of genetically engineered probiotic *E. coli* Nissle 1917 against LPS induced damage in Pb treated rats on: (A) Gastric emptying and intestinal transit. Values are expressed as mean ± SEM (n=6 each group) ** P ≤ 0.01,*P ≤ 0.001 compared to w water (stomach), $^{\#}$P ≤ 0.05, $^{\#\#\#}$P ≤ 0.001 compared to Pb+LPS (stomach), $^{@@}$ P ≤ 0.01, $^{@@@}$P ≤ 0.001 compared to water (proximal Intestine), $^{\$}$P ≤ 0.05, $^{\$\$\$}$P ≤ 0.001 compared to Pb+LPS (proximal Intestine), $^{\%\%}$ P ≤ 0.01 compared to water (middle intestine), $^{\&}$P ≤ 0.05, $^{\&\&\&}$P ≤ 0.001 compared to Pb+LPS (middle intestine), ^P ≤ 0.05, ^^^P ≤ 0.001 compared to water (distal intestine), $^{+}$P ≤ 0.05, $^{+++}$P ≤ 0.001 compared to Pb+LPS (distal intestine), (B) Survival% of rats (n=10 each group); mRNA expression levels of (C) TNF-α, and (D) iNOS in colon (E) Metallothionein in liver. * P ≤ 0.05, ** P ≤ 0.01,***P ≤ 0.001 compared to Water group. ##P ≤ 0.01, ###P ≤ 0.001 compared to Pb+LPS group, $^{\$\$}$P ≤ 0.01,$^{\$\$\$}$P ≤ 0.001 compared to Pb+LPS+*Ec*N-2.**

Gastric emptying and intestinal transit was delayed significantly in Pb+LPS/GalN group compared to control (water) whereas *Ec*N-23 treatment significantly enhanced it as compared to *Ec*N-2 and *Ec*N-22 (**Fig. 4.2**). After 24 hrs of LPS/GalN injection, the survival of rats was decreased to 20% in Pb+LPS/GalN group while it was increased to 80% in rats treated with *Ec*N-23. In colonic content short chain fatty acids (SCFAs) levels were significantly decreased in Pb and Pb+LPS treated groups whereas the treatment with *Ec*N-22 and *Ec*N-23 had brought the SCFAs levels near to normal (**Table 4.1**).

Groups	Control	Pb	LPS	Pb+LPS	*Ec*N-2	*Ec*N-22	*Ec*N-23
Acetate	65.23±2.4	57.44±3.1 a*	63.21±2.2	53.76 ±2.7 a*	57.75 ±2.8	76.42 ±2.6a* b***	85.14±3.7a***b***
Propionate	17.98±1.3	11.52±1.7a*	15.43±1.1	10.66±1.8 a*	13.18±1.4	19.29±1.6b*	24.47±1.5a*b***c*
Butyrate	11.9±0.7	7.3±0.9a*	10.25±1.2	6.4±0.8 a*	8.1±1.4	16±1.3b***	21±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 Pb+LPS**. 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 4.1. Short chain fatty acids (SCFAs) concentration in colonic matter of Pb treated rats after LPS/GalN injection.

4.3.2. Histopathological damage

Histological analysis showed normal tissue architecture while after LPS and Pb+LPS/GalN treatment remarkable changes in histopathology were observed in intestinal sections characterized with inflammatory cell infiltration and eroded epithelium. Moderate recovery of histopathological damage was observed after treatment with *Ec*N-23 (**Fig. 4.3**).

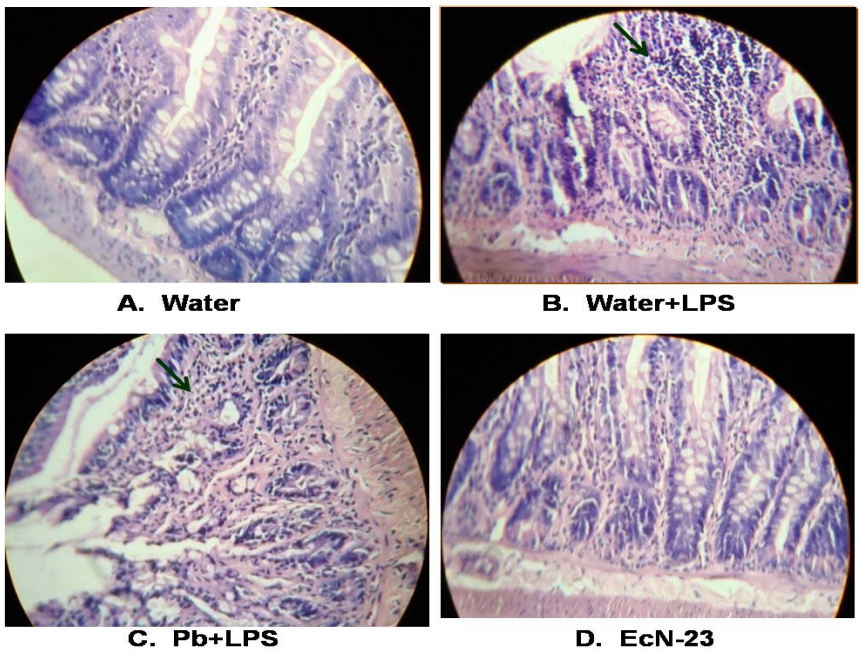


Fig. 4.3. Photomicrograph of intestinal tissue stained with HE (Magnification=40x) (A) Water fed group showing normal tissue architecture, (B) Water+LPS treated group showing loss of tissue architecture, inflammatory cell infiltration and necrosis (black arrow), (C) Pb+LPS treated group also showing loss of tissue architecture, inflammatory cell infiltration and necrosis (black arrow), (D) *Ec*N-23+Pb+LPS showing recovery in tissue architecture.

4.4. Discussion and conclusion

Pb can induce switching of B lymphocytes from producing IgG antibody associated with protection against infectious agents to IgE causing allergic and hypersensitivity responses (Lutz et al., 1999; Basaran and Undeger, 2000; Sun et al., 2003; Karmaus et al., 2005). Pb altered the Th1/Th2 balance by inhibiting the Th1 stimulation essential for maintaining host resistance to infections while promoting the Th2 stimulation (Mccabe and Lawrence, 1991).

The sensitivity of rats to endotoxins can be increased by about 100,000 times above the normal by lead acetate treatment (Selye et al., 1966). In mice by impairing liver metabolism, D-Galactosamine (GalN) further increases sensitivity to LPS-induced damage (Lehmann et al., 1987), therefore LPS/GalN rat model was used (Ewaschuk et al., 2007). High systemic levels of TNF-α induced by LPS triggers the onset of breakdown of intestinal barrier resulting into inflammatory response to endotoxin through translocation of bacteria to the liver (Hassoun et al., 2001). In the present study, in colon high TNF-α transcripts were found in Pb+LPS/GalN treated group.

LPS significantly increases NO levels by increasing iNOS expression, thereafter, NO reacts with superoxide radicals resulting in the formation of peroxynitrite ($ONOO^-$), which is highly reactive and potent oxidizing agent leading to lipid peroxidation (Winter et al., 2005; Paul et al., 2014). LPS also triggers the histamine and platelet activating factor release by mast cell degranulation which further increases the intestinal permeability as well as delayed gastric emptying and intestinal transit (Brown et al., 1998; Winter et al., 2005). In the present study, increase in iNOS expression, NO levels, histamine levels and lipid peroxidation were seen in groups treated with Pb+LPS/GalN.

The neutrophil and macrophages infiltration enhanced by LPS treatment, hence MPO activity was increased in group treated with water+LPS/GalN. Pb intoxication decreased the MPO activity as well as the phagocytic activities of neutrophil and macrophages, thereby disabling the innate immune response (Winter et al., 2005; Paul et al., 2014). Therefore, after Pb+ LPS/GalN treatment MPO activity was found to be less.

Metallothionein (Mt) synthesis is induced in response to Cd toxicity to counter its damaging effects by forming complexes (Garcia-Nino and Pedraza-Chaverri, 2014). In response to LPS, hepatic Mt was induced for stimulating α_1-Acid Glycoprotein which has a protective effect against LPS/GalN induction (Kimura et al., 2003). Treatment with antioxidant is also known to induce Mt induction (Kumar, 2012). In the current study higher Mt levels were observed in the groups treated with Pb, water+LPS, Pb+LPS and *Ec*N-22. Lower Mt levels were observed after *Ec*N-23 treatment as it secretes 2-ketogluconic acid which complexes with Pb, enhancing its excretion thereby decreasing the free Pb content.

Gluconic acid is known to act as a prebiotic (Kameue et al., 2004) produced by both *Ec*N-22 and *Ec*N-23. Gluconic acid can be metabolized by beneficial microbiota in gut resulting in the production of SCFAs, which are known to have anti-inflammatory effects (Cox et al., 2009), thus could protect against pro-inflammatory effects of heavy metals. This is in accordance with our previous study where significantly higher levels of SCFAs such as acetic, butyric and propionic acids were observed in the feacal matter of rats treated with *Ec*N secreting PQQ (Singh et al., 2014) .

The sealing effect of the tight junctions at enterocytes was promoted by *Ec*N (Sonnenborn and Schulze, 2009). Interestingly, LPS/GalN induced damage was lower in groups treated with *Ec*N-2. *Ec*N-22 produces both PQQ as well as gluconic acid whereas *Ec*N-23 produces PQQ, gluconic acid and 2-ketogluconic acid altogether. PQQ protects against the oxidative damage induced by the LPS/GalN whereas 2-ketogluconic acid chelates the Pb, thus *Ec*N-23 increases the survival upto 80% by decreasing the susceptibility to LPS/GalN. In conclusion, the present study demonstrates *Ec*N-23 is effective for countering the immunotoxicity of Pb in LPS/GalN treated rats in colon and liver.