

Research Article



Effective Dose and Duration in Administration of *L. Plantarum* AD3 on Experimentally Induced Uremic Rats

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ABSTRACT

Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Effects of probiotics are strain specific, what that amount should be is not indicated. The minimal effective dose or the level of viable cells of the probiotic strain in terms of CFU/mL/day that demonstrates general health promoting functions. Thirty six male albino rats were divided into six groups (6 rats /dose /week). Group I served as negative control- normal diet, groups II uremic control - exposed acetaminophen interperitonally at the dose of 500mg/kg body weight /day for 10 days, group III, IV, V and VI were different doses group, where 10^7 , 10^8 , 10^9 and 10^{10} CFU/mL of *L. plantarum* AD3 strain were co-administered with acetaminophen (500mg/kg body weight /day for 10 days) at different duration (for 1 week, 2 week and 3 week). Experimental findings suggested that acetaminophen exposure decreased the levels of total count of RBC and Hb% significantly ($p < 0.05$) after 2nd week. Co-administration of *L. plantarum* AD3 resulted in lowered the level of BUN and creatinine when dosed at 10^7 and 10^8 CFU /mL/100g body weight/day, but had no significant difference between the group I, group II and group III. However, significant protection was observed at a dose of 10^9 CFU /mL/100g body weight. However, *L. plantarum* AD3 significantly alleviated the kidney damage in APAP-exposed rats when treated with at minimum dose at 10^9 CFU/mL/100 g of bodyweight/day for 14 days. No obvious difference was observed in the kidneys in between Group IV and Group V samples. No significant change in biochemical and histological parameters was found between 14 days and 21 days exposure with *L. plantarum* AD3. Similarly taking the minimum effective dose, 10^9 CFU /kg body dose optimum for *L. plantarum*AD3for drug induced uremic male rats.

Keywords: Probiotic, uremia, optimum dose.

INTRODUCTION

Popular analgesic and antipyretic drug, especially paracetamol and acetaminophen (APAP), and nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used through the world. Paracetamol and APAP are nephrotoxic drugs. Several *in vitro* and *in vivo* studies showed that analgesics nephrotoxicity is caused by increased ROS in kidney^{1,2}. On the other hand, APAP also undergoes cytochrome P450 mediated activation to the toxic N-acetyl-p- benzoquinone imine (NAPQI) both in liver and kidney³. At therapeutic doses, the quantity of NAPQI formed is relatively small and is detoxified by conjugation with reduced glutathione (GSH)^{4,5}. APAP overdose produces excessive NAPQI and only a part of it can form GSH conjugate with resulting reduction of cellular GSH. The residual part of NAPQI binds to cellular proteins and induces oxidative stress, leading to renal injury. Therefore, searching of defensive pathway that would offer maximum protection against APAP induced renal damages are required. "Enteric dialysis" is an alternative approach for solute removal in uremia is based on the reality that the intestinal barrier functions as a semipermeable membrane. Concentration gradient make solutes disperse from plasma into the lumen when concentration of uremic solute become larger in plasma than lumen and a major parts of uremic solutes become distributed throughout the intestine by binding to ingestible solute-specific sorbents within the gut^{6,7}. This

approach requires large quantities of specific sorbents to be ingested daily. Thus to mitigate uremic solutes using live bacteria which degrade uremic toxins within the gut has been preferable approach of today and is further investigated in the present study^{8, 9, 10}. Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Effects of probiotics are strain specific. The activity of any strain in the gut would only be expressed after its colonial establishment. Potentiality of bacteriotherapy to alleviate uremic status was also noted by other investigators. Mandal et al. (2013)¹¹ reported that oral administration of *Lactobacillus* spp effectively reduced the levels of uremic toxins in dialysis patients. Generally dose of probiotic is very important (as these are dynamic in nature) for exerting any therapeutic effect. It was estimated that 10^9 CFU/mL is an effective dose that can create a sociable environment at intestinal lumen and exert beneficial effect to the host. But effective dose may vary for different ailments. The minimal effective dose or the level of viable cells of the probiotic strain in terms of CFU/mL/day that demonstrates general health promoting functions or well being or specific health claims in target population should be clearly indicated before using it as pharmaceutical vehicles as. Keeping this in view the aim of the present study was to evaluate the optimum dose and duration of antiuremic activities of *L. plantarum* AD3 against acetaminophen induced uremia in rats.



MATERIAL AND METHODS

Selection of Animals and Care

The study was conducted on healthy, adult, male albino rats of Wister strain (Supplied from Ghosh animal, animal foods and animal cages Supplier, Kolkata, India) having a body weight of 100 ± 15 g. They were acclimatized to laboratory condition for 2 weeks prior to experimentation. Animals were housed six per cage in a temperature-controlled room (22 ± 2 °C) with 12-12 h dark-light cycle (8.00-20.00 h light: 20.00-8.00 h dark) at a humidity of 50 ± 10 %. They were provided with standard food (pellet diet) and water ad libitum. The principle of laboratory animal care National Institute of Health USA

Grouping of animals & experimental procedure

The rats were divided into four equal groups as follows

Table 1: Experimental procedure

Groups	Treatment procedure	Duration of <i>L. plantarum</i> AD3 treatment (weeks)
GroupI-NC (Negative Control)	normal diet	6 rats /week
GroupII- UC (Uremic Control)	Acetaminophen induced Uremia	6 rats /week
GroupIII- $D_1D_7, D_1D_{14}, D_1D_{21}$	Co-administration of Acetaminophen + <i>L. plantarum</i> AD3 at 10^7 CFU /mL/100 g of body wt /day	6 rats /week
GroupIV- $D_2D_7, D_2D_{14}, D_2D_{21}$	Co-administration of Acetaminophen + <i>L. plantarum</i> AD3 + 10^8 CFU /mL/100 g of body wt /day	6 rats /week
GroupV- $D_3D_7, D_3D_{14}, D_3D_{21}$	Co-administration of Acetaminophen + <i>L. plantarum</i> AD3 + 10^9 CFU /mL/100 g of body wt /day	6 rats /week
GroupVI- $D_4D_7, D_4D_{14}, D_4D_{21}$	Co-administration of Acetaminophen + <i>L. plantarum</i> AD3 + 10^{10} CFU /mL/100 g of body wt /day	6 rats /week

Acetaminophen induced uremia - acetaminophen exposure (500mg/kg body weight, for 10 day), D_1 , D_2 , D_3 and D_4 for specific dose of *L. plantarum*AD3 (10^7 , 10^8 , 10^9 and 10^{10} CFU /mL/100 g of body wt /day respectively). D_7, D_{14}, D_{21} for different duration (1st week-7 days, 2nd week-14 days and 3rd week- 21 days respectively).

Hematological study

After 3 weeks, animals were sacrificed by diethyl ether anaesthesia. Blood samples were collected by hepatic artery punche under diethyl ether anesthesia, using 21 gauge (21 G) needles mounted on a 5mL syringe (Hindustan syringes and medical devices ltd, Faridabad, India.) into heparin coated sample bottles for analyzed Hematological parameters like RBC count by hemocytometer and hemoglobin (Hb) by standard kit method (Merck, Japan).

Blood Uremia Profile

Biochemical estimation of Blood Urea

The collected blood was centrifuged and plasma fraction was separated. Urea level of plasma was measured by commercially available standard Blood Urea Kit (Merck, Japan) by Semi autoanalyser (Merck, Japan) by standard

(NIH, 1985) guideline was followed throughout the duration of experiment⁸ and our Institute Ethics committee approved the experimental protocol according to the CPCSEA guidelines (CPCSEA Registration No-1905/PO/Re/S/2016/CPCSEA).

Grouping of animals and experimental procedure

Acute toxicity study was performed according to Organization of Economic Co-operation and Development (OECD) guidelines-423 (acute toxic class method). Experiments of acetaminophen injection were done at the dose of 500mg/kg body weight /day for 10 days was chosen for studying biochemical alterations in acetaminophen induced uremic rats.

protocol for photometric determination of urea according to the Urease GLDH method¹².

Biochemical estimation of Blood Creatinine

The collected blood was centrifuged and plasma fraction was separated. Creatinine level of plasma was measured by commercially available standard creatinine Kit (Merck, Japan) by Semi autoanalyser (Merck, Japan) by standard protocol for photometric determination of creatinine based on Jaffe kinetic method without deproteinization¹³.

Statistical analysis

All the values are expressed as mean \pm SE (n = 6). Significant differences between the groups were determined with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) for Windows using one-way analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). A difference was considered significant at the $P < 0.05$ level.

RESULT AND DISCUSSION

Blood uremic profile

According to the result we found that acetaminophen exposure was significantly decreased the levels of total count of RBC and Hb% after 2nd week comparison to



negative control (Group I). It was observed that total count of RBC and Hb% were significantly altered when *L.plantarum* AD3 was dosed at 10^9 and 10^{10} CFU /mL/100g body weight/ day in comparison to uremic group (Group II). However, there was no significant difference in between GroupV and Group VI (at 10^9 and 10^{10} CFU /mL/100g body weight/ day dose respectively) (in Fig. 1.1.III and IV). In time dependent study at the effective dose of 10^9 CFU//mL/100g body weight/ day of *L.plantarum* AD3, maximum protection was observed after the treatment for 2nd week (14 days). No significant change was observed even with increasing the time of *L. plantarum* AD3 treatment for 3rd week (21 days) respect to 2nd week (Fig 1.1 V and VI).

Acute exposure of acetaminophen, a widely used analgesic and antipyretic drug, causes severe renal damage. No specific agent has been reported so far that plays any beneficial role in the organ pathophysiology. These findings let us to investigate the appropriate dose and duration involved in the nephro intoxicative effect of isolated urease positive probiotic LAB strain, against acetaminophen induced renal pathophysiology. Acetaminophen nephrotoxicity results from its highly reactive and toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which destroy proteins in the S3 segment of the proximal tubule and that in turn, initiates the damage of renal tubular¹⁴. The fundamental function that blood cells perform together with the susceptibility of this highly proliferative tissue to intoxication by xenobiotics makes the hematopoietic system unique as a target organ. Erythrocytes, leukocytes and platelets were produced at a turnover rate of about 1-3 million per second in a healthy human adult, and these values can be distorted in certain physiological or pathological states during hemolytic anemia or suppressive inflammation¹⁵. Certain drugs including alkylating cytotoxic agents can

also affect blood cell development rates and the normal range of hematological parameters. In the present study, it was revealed that the high dose of acetaminophen intraperitoneally for 10 days were significantly decreased Hb levels and also lowered of total RBC counts (Figure 1.1). The decreased Hb level was supposed due to be destruction of RBCs and lower total RBC count due to hemolytic anemia or suppressive inflammation. Co-administration of *L. plantarum* AD3 with acetaminophen for minimum 2 weeks, at a dose of 10^9 CFU /mL/100g body weight/day, restored the Hb levels as well as total RBC counts than the acetaminophen-induced uremic group (II). However, this study showed that the bacteria could reverse the hematotoxic effect of acetaminophen, with resulting improvement of hematopoiesis.

Experimental findings suggested that acetaminophen exposure (500mg/kg body weight, for 10 day) was significantly increased the levels of blood urea nitrogen (BUN) and creatinine after 14 days in uremic group (group II) compared to negative control (group I) (Figure 1.1 III and IV). Co-administration of *L. plantarum* AD3 resulted in lowered the level of BUN and creatinine when dosed at 10^7 and 10^8 CFU /mL/100g body weight/day, but had no significant difference between the group I, group II and group III (Fig 1.1). However, significant protection was observed at a dose of 10^9 CFU /mL/100g body weight (as presented in the Fig. 1.1). Similarly the dose of 10^9 CFU /mL/100g body weight/day optimum for both bacteria in time dependent study. In this study the maximum elevation was observed after the treatment for 14 days for *L. plantarum* AD3 and the result did not change much even with increasing the time of bacteria supplementation. This drug-induced nephrotoxicity is frequently associated with marked elevations in blood urea nitrogen, serum creatinine and acute tubular necrosis^{16, 17}.

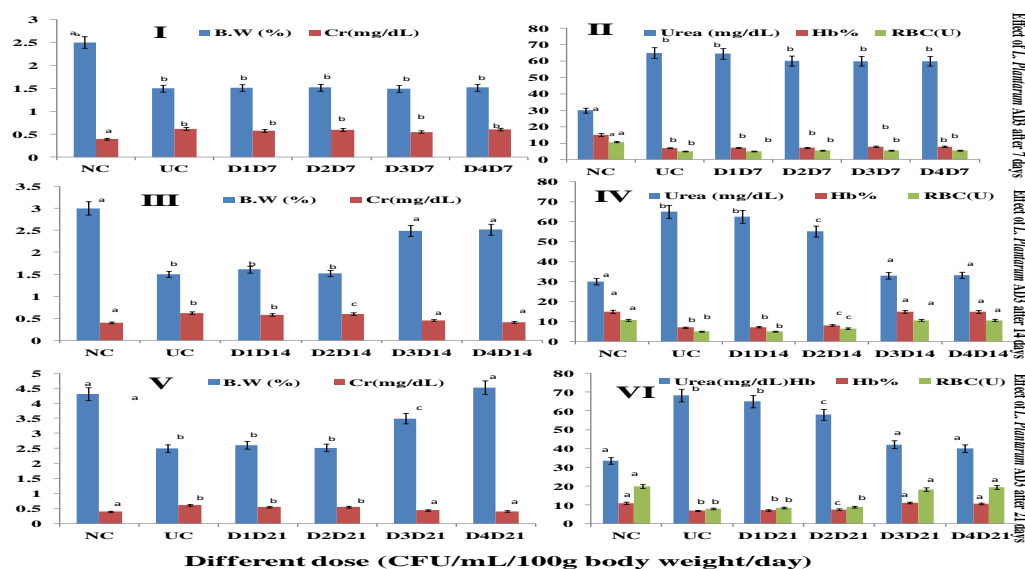


Figure 1.1: Dose and time dependent effect of *L.plantarum* AD3 on uremic parameters in APAP induced toxicity in the blood of the experimental rats. In this figure-I and II show effect of 7 days administration of *L.plantarum* AD3 on uremic rats. In figure-III and IV show effect of 14 days administration of *L.plantarum* AD3 on uremic rats. NC – negative control

group, UC- uremic group, In the groups D1, D2, D3 and D4- for specific dose of *L.plantarum* AD3(10^7 , 10^8 , 10^9 and 10^{10} CFU /mL/100 g of body wt /day respectively. D7D14D21- for different duration (7 days, 14 days and 21 days respectively). In all cases data are expressed as Mean \pm SE (n=6). ANOVA followed by multiple two tail t-test. Bars with different superscripts (a, b, c) differ from each other significantly ($p < 0.05$)

In the present study, administration of a nephrotoxic dose of acetaminophen to rats resulted in progression of oxidative stress harmful to renal tissues. Acetaminophen-induced nephrotoxicity showed a significant ($p < 0.05$) increase in the plasma urea and creatinine concentrations in Group II (acetaminophen induced uremic) rats when compared with the negative control group (Group I) (Fig 1.1). Moreover, oral administration of *L. plantarum* AD3 for 2 weeks, in the dose of 10^9 CFU /mL/100g body weight/day significantly ($p < 0.05$) decreased plasma urea and creatinine, when compared with Group II. The intestinal mucosal surface functions as a semi-permeable membrane. Driven by the concentration gradient, solutes with higher concentration in circulating blood diffuse from plasma into the intestine, and a large portion of uremic solutes are differentially distributed within the bowel.

Histological parameter

Kidney histological study was used to determine the protective effect of *L.plantarum* AD3 on APAP-induced injury (Fig.1.2) at different dose for 14 days. APAP treatment (UC) caused several visible histological changes. The renal sections showed extensive tubular damage by presence of necrotic epithelial cells. Tubular degeneration, necrosis, cell swelling, mononuclear cell infiltration and degenerated organelles were also observed in the kidney following the APAP exposure. Some epithelial cells were found damaged in the tubular lumen (Fig. 1.2). However, *L.plantarum* AD3 significantly alleviated the kidney damage in APAP-exposed rats when treated with at minimum dose at 10^9 CFU/mL/100 g of bodyweight/day for 14 days. No obvious difference was observed in the kidneys in between Group IV and Group V samples (Fig. 1.2 E and F).

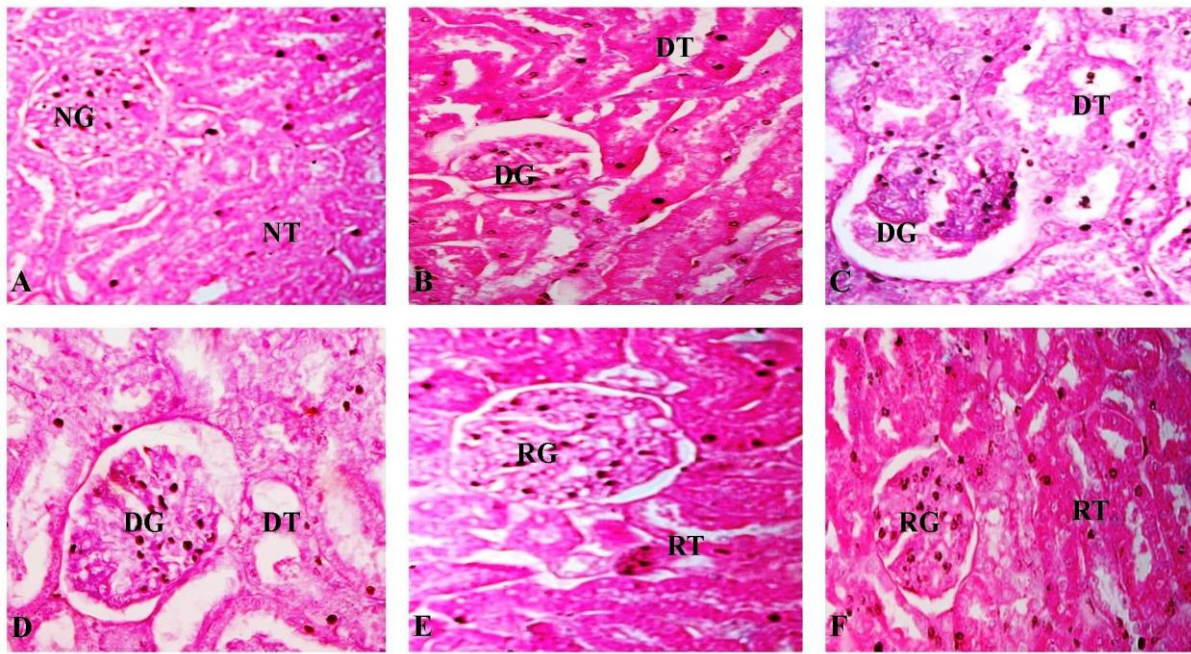


Figure 1.2 Histological study of *L. plantarum* AD3 treatment group at different dose for 14 days comparison to control groups.(A): Showing normal histology of kidney of negative control group of rats (NC). Normal Glomerulus (NG) and renal tubules (NT) are well organized in histoarchitecture. (B) Showing severe disorganization of rat kidney after acetaminophen injection of 500mg/kg body weight to uremic control group rats (UC). Damaged Glomerulus (DG) and dilated renal tubules (DT) are seen. (C) Showing acetaminophen induced uremic rat kidney (500mg/kg body weight) with treatment by *L. plantarum* AD3 at the dose of 10^7 CFU/mL/100 g of bodyweight/day for 14 days to group III rats showing damaged glomerulus (DG) and dialated tubule (DT).and.(D) Showing acetaminophen induced uremic rat kidney (500mg/kg body weight) with treatment by *L. plantarum* AD3 at the dose of 10^8 CFU/mL/100 g of bodyweight/day for 14 days to group IV rats showing damaged glomerulus (DG) and dialated renal tubule (DT).and (E) Showing acetaminophen induced uremic rat kidney (500mg/kg body weight) with treatment by *L. plantarum* AD3 at the dose of 10^9 CFU/mL/100 g of bodyweight/day for 14 days to group V rats showing control like histology with reorganized glomerulus (RG) and renal tubule (RT).and (F) Showing acetaminophen induced uremic rat kidney (500mg/kg body weight) with treatment by *L. plantarum* AD3 at the dose of 10^{10} CFU/mL/100 g of bodyweight/day for 21 days to group VI rats showing organized glomerulus (RG) and renal tubule (RT).

Treatment by *L. plantarum* AD3 in Group IV at minimum dose of 10^9 CFU/mL/100 g of bodyweight/day for 14 days showed an improvement and rearrangement of these cells comparison to Group III and Group IV (Fig 1.2). Orally administration of urease positive probiotic *L. plantarum*AD3 at high dose showed control like histoarchitecture of kidney. No significant change in biochemical and histological parameters was found between 14 days and 21 days exposure with *L. plantarum*AD3 (Fig 1.3).

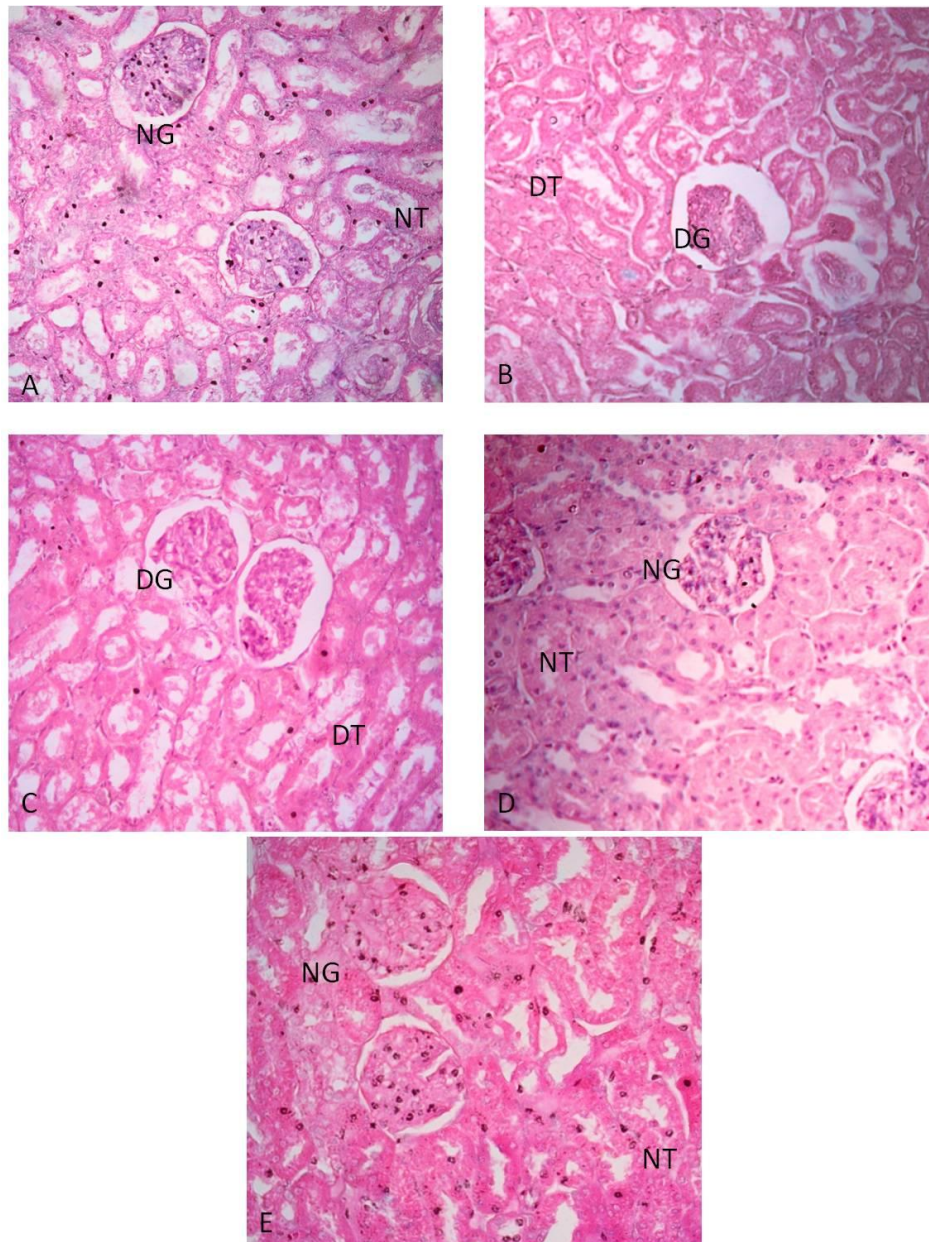


Figure 1.3 Histological study of *L. plantarum* AD3 treatment group at 10^9 CFU/mL/100 g of bodyweight/day for different duration comparison to control groups. (A) Showing normal histology of kidney of control group of rats. Normal Glomerulus (NG) and renal tubules (NT) are well organized in histoarchitecture. (B) Showing severe disorganization of rat kidney after acetaminophen injection of 500mg/kg body weight to Group II rats. Damaged Glomerulus (DG) and dilated renal tubules (DT) are seen. (C) Showing acetaminophen induced uremic rat kidney (500mg/kg body weight) with treatment by *L. plantarum* AD3 at the dose of 10^9 CFU/mL/100 g of bodyweight/day for 7 days to group III rats showing control like histology with normal glomerulus (NG) and renal tubule (NT). (D) Showing acetaminophen induced uremic rat kidney (500mg/kg body weight) with treatment by *L. plantarum* AD3 at the dose of 10^9 CFU/mL/100 g of bodyweight/day for 14 days to group III rats showing control like histology with normal glomerulus (NG) and renal tubule (NT) and (E) Showing acetaminophen induced uremic rat kidney (500mg/kg body weight) with treatment by *L. plantarum* AD3 at the dose of 10^9 CFU/mL/100 g of bodyweight/day for 21 days to group III rats showing normal glomerulus (NG) and renal tubule (NT) like negative control.

CONCLUSION

In this study biochemical analysis of uremic markers were measured to confirm the nephrotoxicity. The histology of kidney also showed a severe disorganization after treatment of APAP (500mg/Kg body weight for 10 days). And co- treatment with *L.plantarumAD3* strain at 10^9 CFU/mL/100 g of bodyweight/day for 14 days could restore the damage. Control like histoarchitecture of kidney was also observed (Fig 4.2d). Thus the lower dose (1×10^9 CFU /mL/100g body weight/ day for 14 days) was suggested for isolated probiotic strain than the higher dose (1×10^{10} CFU /mL/100g body weight/ day) as effective therapeutic dose according to ICMR-DBT guide line 2011. It was evident that the bacteria supplementation at a dose of 10^9 CFU /mL/100g body weight/ day/ 14 days has curative role against acetaminophen induced uremia. The mode of action of uremia prevention may probably due to its high urease enzyme production property as well as antioxidant property (discussed in chapter2). In the case of group treated with *L. plantarum* AD3 at a dose of 10^9 CFU /mL/100g body weight/ day duration for 2 weeks showed significant reduction of uremic profiles.

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