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Therapeutic potential of *Lactobacillus ingluviei* ADK10, a newly established probiotic organism against acetaminophen induced uremic rats*

Arpita Mandal[1], Tanmay Paul[2], Suchismita Roy[1], Shreya Mandal[1], Shrabani Pradhan[1], Koushik Das[1], Keshab Chandra Mondal[2]** & Dilip Kumar Nandi[1]

[1]*Department of Microbiology, Nutrition, and Human Physiology, Raja N L Khans Women's College, Midnapore – 721102, West Bengal, India*
[2]*Department of Microbiology, Vidyasagar University, Midnapore – 721102, West Bengal, India;*
e-mail: kcmondal@mail.vidyasagar.ac.in

Abstract: In the present study, *Lactobacillus ingluviei* ADK10 (Acc. No. JQ395039) from intestinal origin was tested for its probiotic characteristic as well as uremia ameliorating activity on acetaminophen induced uremic rats. The results revealed that *L. ingluviei* ADK10 was able to tolerate pH 3.0–9.0 and 0.5% bile salt along with good hydrophobicity (67%) and adherence index with Ht-29 cell line on 258/100 cells. It was susceptible to 20 antibiotics. The organism was able to degrade food ingredients, like starch and milk proteins. The strain showed significant growth inhibition of *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Shigella dysentery*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (average diameter of 10 mm). The therapeutic potentiality of this probiotic bacterium was tested against acetaminophen induced uremic rats. It was found that supplementation of *L. ingluviei* ADK10 for 14 days with food reduced severe increase of uremic profiles, such as blood urea (85%), creatinine (68%) and uric acid (41%) in comparison to the uremic rats. Moreover, during the feeding of rats with probiotic strain at a dose of 1×10^9 bacteria, reduction of enterobacteria in faeces was observed. Our studies indicated that *L. ingluviei* ADK10 could be used as a health-promoting probiotic along with antiuremic efficacy.

Key words: probiotic properties; *Lactobacillus ingluviei* ADK10; antiuremic efficacy.

Introduction

Renal insufficiency leads to uraemia and each year, the number of patients with chronic kidney failure increases by a surprising 11% (Jain et al. 2009). Due to the global shortage of kidney donors for kidney transplants as well as due to high costs linked with transplant surgery and high probability of organ rejection, most patients worldwide have very few options for effective treatment after kidney failure. There is great necessity in the mission for an unconventional, affordable therapy for patients who cannot afford expensive dialysis or kidney transplant to keep them alive.

"Enteric dialysis" is an adaptive physiological process for removal of solutes from the body. High concentration gradients can facilitate diffusion of solutes from plasma to intestinal lumen. Recent studies indicated that uremic toxins like urea, creatinine, etc., can excrete through enteric dialysis (Sparks 1979). The urease producing bacteria on mucosal layer of intestine may accelerate the dialysis process of uremic toxins. Probiotics and prebiotics evaluate the impact on solute concentration in serum or on their faecal or urinary excretion (Hida et al. 1996). The live bacteria which degrade uremic toxins within the gut have been an acceptable therapy of today (Hida et al. 1996; Prakash & Chang 1996).

The first option aims at modifying the intestinal flora to refrain generation of toxins, either by prebiotics, which are non-digestible compounds beneficially modifying the composition and/or function of the intestinal flora; or by probiotics, which are bacteria administered as food components or supplements providing specific benefits themselves reducing urea alone (Bliss et al. 1996; Ranganathan et al. 2006). Probiotics and prebiotics evaluate the impact on solute concentration in serum or on their faecal or urinary excretion (Hida et al. 1996). Probiotics may have a substantial impact on human welfare even also in uremia and support the antioxidative system, which breaks down during acetaminophen overdose.

In this context, present study was conducted to evaluate potential health beneficial role of a urease positive lactic acid bacteria against uremic rats. Our pre-

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** Corresponding author

vious study (Mandal et al. 2013b) demonstrated that feeding with *Sporosarcina pasteurii* attenuates blood urea-nitrogen levels and improves in the life span of uremic animals. The objective of this study was to investigate the probiotic characterization of *Lactobacillus ingluviei* ADK10 (Mandal et al. 2013a), isolated from the intestinal tract of chicken and to evaluate the bacterium as a remedy for uremia in acetaminophen induced uremic rats.

Material and methods

Bacterial strains and culture conditions

Indicator bacteria (*Escherichia coli* ATCC 8739, *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC 25093, *Shigella dysentery*, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 10145) used for antimicrobial assays were cultured in tryptone soy agar and stored under aerobic condition at 4 °C. *L ingluviei* ADK10 (GenBank Acc. No. JQ395039) was isolated in our laboratory and stored under anaerobic condition at –20 °C.

Probiotic characteristics

Composition of basal media. Basal media for *L. ingluviei* ADK10 contained several components as follows: 1.0% peptone, 0.8% meat extract, 0.4% yeast extract, 2.0% glucose, 0.5% sodium acetate trihydrate, 0.1% polysorbate 80 (also known as Tween 80), 0.2% dipotassium hydrogen phosphate, 0.2% triammonium citrate, 0.02% magnesium sulfate heptahydrate and 0.005% manganese sulphate tetrahydrate (pH 6.5).

Analytical studies of the organism. Growth of the organism at 37 °C was tested in different medium pH (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10), NaCl concentration (1, 2, 3, 4, 5, 8, 9 and 10% [w/v]), bile salt concentration (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 g sodium taurocholate/100 mL), and phenol level (0.1–1.0 g phenol/100 mL) (Erkkilä & Petäjä 2000).

Survival under conditions simulating the human gastrointestinal tract. Resistance to low pH was tested as described previously (Charteris et al. 1998). Tolerance to small intestine conditions was tested (Maragkoudakis et al. 2006).

Cell surface hydrophobicity assay. Hydrophobicity was determined using bacterial adherence to hydrocarbons by xyline (Thapa et al. 2004).

Antibiotic resistance study. Antibiotic resistance study of the bacteria was done using HiMedia Icosha Disc. The plate was supplemented with 1 mL bacterial inoculum (inoculum concentration 5×10^5 CFU/mL), discs were incubated at 37 °C overnight, and diameters of the zone of inhibition around the discs were measured.

Antimicrobial activity and nature of antimicrobial substances. Twenty-four hours culture of *L. ingluviei* ADK10 (log phase) was taken and turbidity was adjusted to 0.5 Mc Farland (corresponding to 10^8 CFU/mL) with phosphate buffer. One mL cell-free culture supernatant of *L. ingluviei* ADK10 was retained as untreated filtrate. To determine the organic acid function, 1 mL cell-free culture supernatant was adjusted to pH 6.5. In order to test the heat sensitivity, 1 mL cell-free culture supernatant was incubated at 100 °C for 15 min and treated with protease-K (Jones et al. 2008). The antimicrobial activity of all samples was tested using the agar-well assay against 24-hour old indicator organisms (Rammelsberg & Radler 1990).

Detection of enzymatic activities. Modified MRS agar containing skimmed milk (HiMedia Laboratories Pvt. Ltd., India), tributyrin and soluble starch was used for detecting the protein, lipid and starch digesting capabilities of selected strain, respectively. The digesting capability of the tested strain was classified as positive when the diameters of clear zone were larger than 1 mm. Each assay was performed in triplicate (Musikasang et al. 2009).

Urease assay. The urease activity was determined for *L. ingluviei* ADK10 by measuring the amount of ammonia released from urea according to the phenol-hypochlorite assay method (Natarajan 1995).

In vitro cell adherence assay. Adherence of *L. ingluviei* ADK10 to HT-29 cells (human colon adenocarcinoma grade II cell line) was examined as described previously (Chauviere et al. 1992). The HT-29 monolayer cells were prepared on glass cover slips and placed in six-well plates, washed twice with phosphate-buffered saline (pH 7.4). One mL *L. ingluviei* ADK10 (10^8 CFU/mL) and 1 mL cell line culture medium were added to each well of the tissue culture plate, and the plate was incubated at 37 °C in 5% CO_2. After incubation for 2 h, the mono layers were washed four times with sterile phosphate-buffered saline, fixed with methanol for 30 min, and examined by phase contrast microscopy. The adherence index was determined from 20 random microscopic fields of adhering *L. ingluviei* ADK10 to 100 HT-29 cells.

Evaluation of the antiuremic effect

Grouping of animals and experimental procedure. Healthy, adult, male albino Wistar strain rats (18) weighing 100 ± 15 g (supplied by Ghosh animal, Animal Foods and Animal Cages Supplier, Kolkata-54) were used. The principle of laboratory animal care of National Institute of Health, USA, guideline was followed throughout the duration of experiment (Olert et al. 1993). The Institute Ethics committee approved the experimental protocol. Animals were randomized and divided into three groups (NC, U, and UP) of six animals each. Group NC served as untreated control and was injected with distilled water 1 mL/100g body weight daily for 7 days. Groups UP and U animals were treated with 500 mg/kg body weight of the acetaminophen by intraperitoneal injection for 7 days, respectively, for inducing uremia.

Formulation preparation. Food balls were arranged by the casein-based diet with*L. ingluviei* ADK10, sterile 10% honey and milk mixture (Ranganathan et al. 2006). The formulation was stored in a –70 °C freezer in aseptic conditions. While no microbial additives were given to rats of NC and U groups, UP group was administered with 1×10^9 CFU of *L. ingluviei* ADK10 for 14 days. After 14 days all experimental animals from all groups were sacrificed and blood, liver and kidney were collected.

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 semiautoanalyser by standard protocol for photometric determination of urea according to the Urease GLDH method (Burtis & Ashwood 1999).

Biochemical estimation of blood creatinine. Creatinine level of plasma was measured by commercially available standard blood creatinine kit (Merck, Japan) by semiautoanalyser (Merck, Japan) by standard protocol for photometric determination of creatinine based on Jaffe kinetic method without deproteinization (Sabbagh et al. 1988).

Biochemical estimation of blood uric acid levels. Enzymatic determination of blood uric acid was performed by

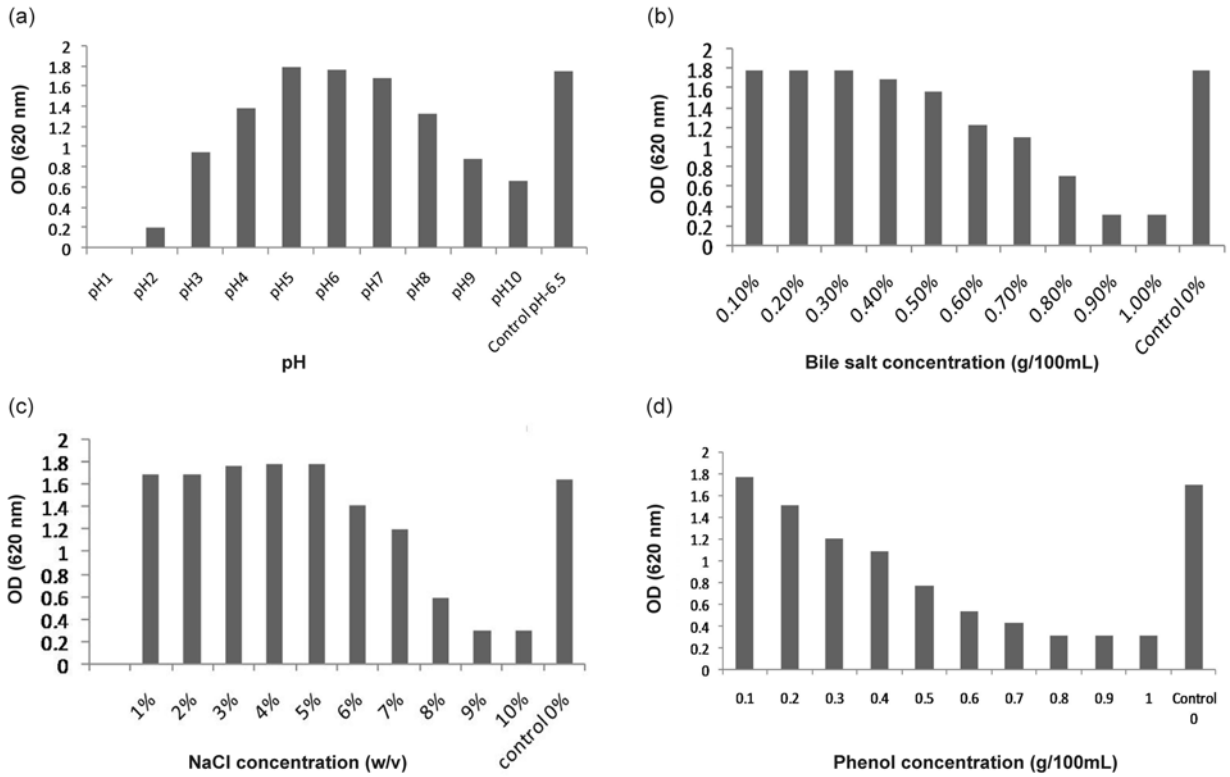

Fig. 1. Probiotic characterization of *L. ingluviei* ADK10 as a survival at different pH values (a), bile salt concentration (b), NaCl concentration (c), and phenol level (d).

uric acid kit (Merck, Millipore). End product of the reaction is quinoneimine and was measured colorimetrically at 546 nm (Fossati et al. 1980) using the diagnostic reagent kit manufactured by Merck Japan.

Limited analysis of faecal enteric bacteria

Limited analysis of faecal microbiota was performed for rats from all groups. Fresh faecal samples were obtained from individual animals at baseline (before treatment) and after 14 days of feeding. Samples were stored at –20 °C for analysis. Faecal mass was re-suspended in physiological peptone saline at an approximate concentration of 0.02 g/mL. Serial dilutions in peptone saline were prepared, and 0.1-mL aliquots from dilutions of 10^{-5} and 10^{-7} were placed on eosin methylene blue agar (Hi-media, India) to obtain enteric bacteria count.

Statistical analysis

Analysis of variance (ANOVA) followed by a multiple two-tail t test with Bonferroni modification was used for statistical analysis of the collected data. Differences were considered significant when $p < 0.05$.

Results and discussion

Tolerance to inhibitory substances

In the present study, *L. ingluviei* ADK10 was evaluated as a potential probiotic for uremia in acetaminophen induced rat model. Before evaluating its antiuremic efficacy, the probiotic characteristics of the bacteria were first studied.

This bacterium is able to grow at pH 3.0 and its significant growth was obtained from pH 5.0 to 9.0 (Fig. 1a). According to Charteris et al. (1998), probiotic bacteria should be tolerated at pH 3.0 for several minutes, while viable count will be affected at slightly high acidic pH. Bile tolerance is an important characteristic for the survival and growth of bacteria in the intestinal tract (Charteris et al. 1998). It has been found that studied bacterium can survive up to 0.8% (w/v) bile salt, which is within the limit of intestinal content (Fig. 1b). *L. ingluviei* ADK10 could tolerate up to 8% NaCl (Fig. 1c). *L. ingluviei* ADK10 further tolerates relatively lesser amount of phenol [0.7% (w/v) shown in Figure 1d]. Tolerance to phenol is an important probiotic property because phenols are accumulated in the intestines by bacterial metabolism of aromatic amino acids and endogenous proteins (Gilliland & Rich 1990).

Survival of bacteria under conditions simulated gastric juice

In simulating gastric juice, the viability of the *L. ingluviei* ADK10 was significantly higher (Table 1). The strain examined in this study could survive well in pancreatin solution at pH 8.0 as even after 4 h of exposure, retained higher viability. Testing the survival of bacteria in simulated gastrointestinal tract conditions *in vitro* may have value in predicting the actual survival of a strain *in vivo* when consumed in a non-protected way. Our findings on the viability of the *L. ingluviei* ADK10 in the presence of pepsin at pH 2 are in agreement with the data reported previously (Charteris et al. 1998). *L. ingluviei* ADK10 survives well in the simulating small intestine environment. Most studies so far have shown majority of the strains survived well under

Table 1. *L. ingluviei* ADK10 survival under the human gastrointestinal tract conditions.[a]

Pepsin at pH 2 (log CFU/mL)			3% Oxgall (log CFU/mL)	
1h	2h	3h	1h	4h
9.34±0.52*	9.14±0.6*	8.74±0.23*	9.04±0.12*	8.94±0.56*
In control condition (log CFU/mL)				
9.44±0.20*	9.34±0.32*	9.31±0.51*	9.38±0.22*	9.37±0.27*

[a] In all cases data are expressed as mean ± SE ($n = 3$). ANOVA followed by multiple two tail t-test. Asterisks represent specific data no significantly different from each other ($p < 0.05$).

Table 2. The antimicrobial activity of cell-free culture supernatant (CFCS) of *L. ingluviei* ADK10 strain by several treatments.[a]

Indicator strains	Inhibition zone (mm)		
	CFCS	CFCS treated with proteinase-K	Heat treated CFCS (100 °C, 15 min)
Escherichia coli	+	+	+
Shigella dysenteriae	++	++	++
Klebsiella pneumoniae	++	++	++
Staphylococcus aureus	++	++	++
Pseudomonas aeruginosa	++	++	++
Bacillus subtilis	++	++	++

[a] Symbols refer to the size of the inhibition zone diameter observed with growing cells: +, 5 mm; ++, 10 mm.

Table 3. Antibiotic sensitivity profile of *L. ingluviei* ADK10.

No.	Antibiotic	Concentration	Susceptible/registrant
1.	Cephalothin	30 mcg	Susceptible
2.	Clindamycin	2 mcg	Susceptible
3.	Co-Trimoxazole	25 mcg	Susceptible
4.	Erythromycin	15 mcg	Susceptible
5.	Gentamycin	10 mcg	Susceptible
6.	Ofloxacin	5 mcg	Susceptible
7.	Penicillin	10 units	Susceptible
8.	Vancomycin	30 mcg	Susceptible
9.	Ampicillin	10 mcg	Susceptible
10.	Chloramphenicol	30 mcg	Susceptible
11.	Oxacillin	1 mcg	Susceptible
12.	Linezolid	30 mcg	Susceptible
13.	Azithromycin	15 mcg	Susceptible
14.	Amikacin	30 mcg	Susceptible
15.	Clarithromycin	15 mcg	Susceptible
16.	Teicoplanin	10 mcg	Susceptible
17.	Methicillin	5 mcg	Susceptible
18.	Amoxyclave	30 mcg	Susceptible
19.	Novobiocin	5 mcg	Susceptible
20.	Tetracycline	30 mcg	Susceptible

small intestine conditions, suggesting a potential recuperation of the initial levels during the passage of the small intestine (Charteris et al. 1998).

Hydrophobicity test

L. ingluviei ADK10 showed hydrophobicity values of 67%. Regarding the percentage affinity to *n*-hexane, the bacteria are classified by the scale: <10% hydrophilic, 10–29% medium hydrophilic, 30–54% medium hydrophobic, >55% highly hydrophobic. Thus hydrophobicity of *L. ingluviei* ADK10 was highly hydrophobic and possesses greater value than some known cultures like *Lactobacillus plantarum* ATCC8014, *L. pentosus* ATCC8041, *L. casei* NCIMB 3254, and *L. delbrueckii* NCIM2025 for which the hzdrophobicity values have been found to be 5.5, 6.5, 6.2 and 3.7%, respectively. As the hydrophobicity of the cell increases, the level of adhesion also increases. Only for hydrophobic microorganisms surface hydrophobicity is correlated to adhesions (Rijnaarts et al. 1993).

Antimicrobial activity

L. ingluviei ADK10 showed antimicrobial activity against potential human pathogens by producing acid

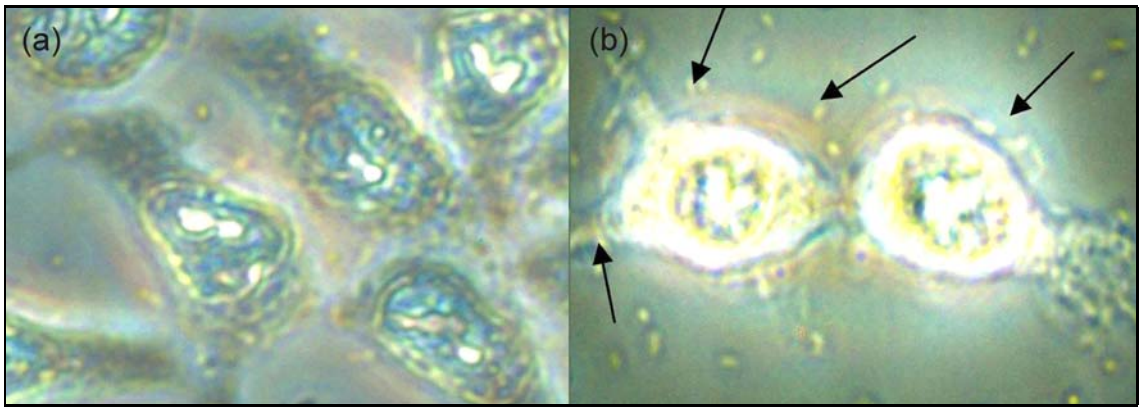

Fig. 2. Adhesion of *L. ingluviei* ADK10 on HT-29 cell cultures observed under phase contrast microscope (40×) without staining. (a) Blank HT-29 cell line, (b) *L. ingluviei* attached with HT-29 cell line (black arrows).

Table 4. Analysis of faecal enteric bacteria.[a]

Groups[b]	Colony No. at 10^{-5} dilution on EMB agar media at baseline	Colony No. at 10^{-5} dilution on EMB agar media at the end of treatment
NC	71 ± 6.1*	77 ± 4.4*
U	78 ± 3.1*	80 ± 7.6*
UP	76 ± 9.2*	41 ± 3.7**

[a] In all cases data are expressed as mean ± SE ($n = 6$). ANOVA followed by multiple two tail t-test.
A single asterisk represents specific data no significantly different from each other, whereas double asterisk means specific data having significantly difference from other $p < 0.05$). CFU, colony-forming unit; EMB, eosin methylene blue.
[b] Groups are as follows: NC, control; U, acetaminophen-receiving control; and UP, Sp treatment followed by acetaminophen.

and organic compounds. It was noted that lactic acid and acetic acid are the major acidic compounds in the culture broth. Table 2 lists the zone of inhibition. Probiotics are known for their production of various antimicrobial compounds, such as organic acids, ammonia, hydrogen peroxide and bacteriocins (Ouwehand & Vesterlund 2004). The production of these compounds by lactic acid bacteria and other intestinal microflora is probably one of the most important mechanisms responsible for the antagonistic phenomenon against pathogenic organisms (Gomes et al. 2006) and therefore it is vital to check this property in probiotic candidates.

Antibiotic resistance
This work reports the susceptibility patterns of *L. ingluviei* ADK10 against all of twenty antibiotics in Icosha disc (Hi-media, India). *L. ingluviei* ADK10 was susceptible to all of the antibiotics tested (Table 3).

Starch, protein, and lipid digesting capabilities
The agar plate assays were used to study digesting capability of the *L. ingluviei* ADK10. In this study, sterilized skimmed milk, tributyrin and soluble starch were used for detecting protein, lipid and starch digestion capabilities, respectively. *L. ingluviei* ADK10 exhibited starch and casein of skimmed milk digestibility which means amylase and protease activity *in vitro*. The beneficial effects of enzymes in the lactic acid bacteria show the importance of enzymatic activities in bacterial strains of probiotics. Utilization of some lactic acid bacteria strains in nutrition is due to their production of enzymes, such as α-amylase, phytase, lecitinase, lipase, and/or protease. Bacteria, which are able to digest starch, protein and lipid, could enhance the good health of animals and human (Duangjitcharoen et al. 2008).

Urease assay
L. ingluviei ADK10 produced a large amount of urease, about 188 unit urease activities (U/mL). The tested bacteria use urea as sole nitrogen source. Thus produced ammonia become utilized within intestine and making no adverse effect on experimental animals.

In vitro cell adherence assay
The adhesion of the strain to HT-29 cells was examined by phase contrast microscopy. Adherence index of *L. ingluviei* ADK10 was 258 bacteria/100 cells. Bacterial adhesion to epithelial cells has been considered as one of the selection criteria for probiotic strains. In the present investigation, the numbers of bacteria adhering to HT-29 cell line were therefore measured. *L. ingluviei* ADK10 shows highly adhesive property in comparison with other probiotic bacteria (Gopal et al. 2001). The adherence index of *L. ingluviei* ADK10 strain demonstrated a considerable adherence according to the report of Jacobsen et al. (1999) and adhering of *L. ingluviei* ADK10 to Ht-29 is shown in Figure 2.

Nephrotoxic effect of acetaminophen and protection by L. ingluviei ADK10 in experimental animals
Acetaminophen exposure significantly increased plasma

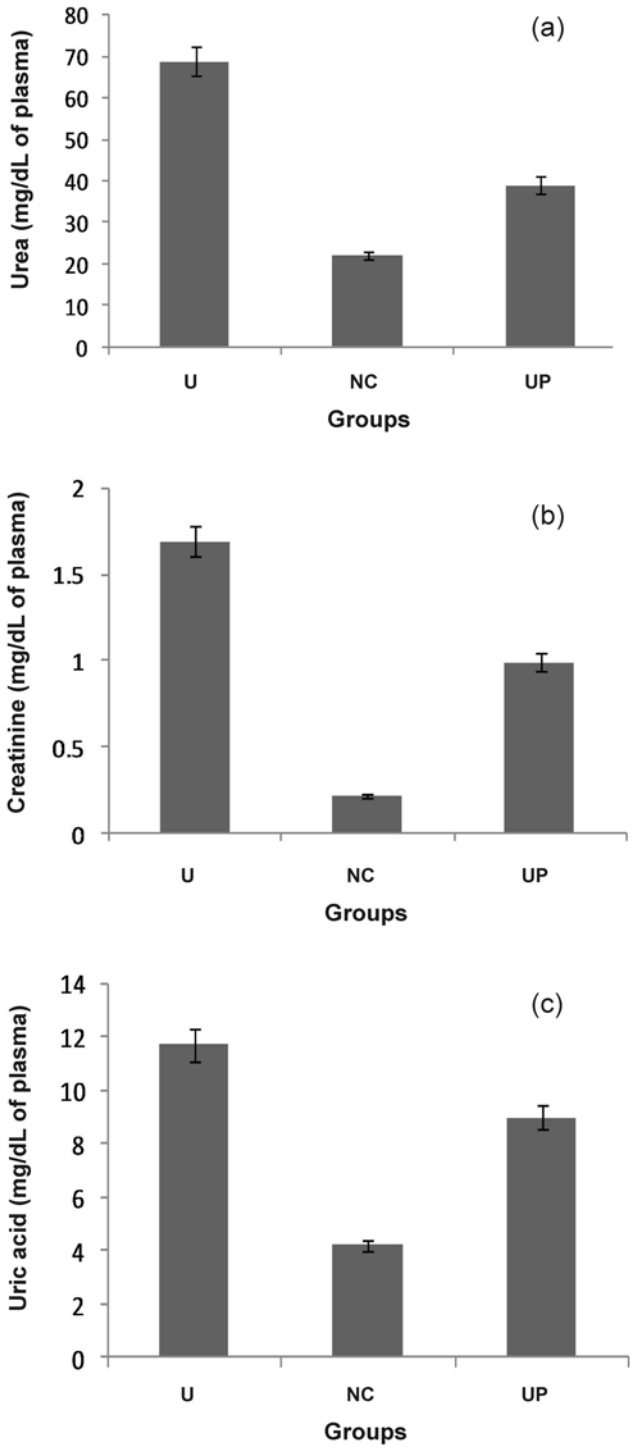

Fig. 3. Effect of acetaminophen and after then *L. ingluviei* ADK10 treatment on plasma urea (a), creatinine (b), and uric acid (c). In all cases data are expressed as mean±SE (n=6).

level of blood urea-nitrogen (Fig. 3a), creatinine (Fig. 3b), and uric acid level (Fig. 3c), However, *L. ingluviei* ADK10 treatment to acetaminophen administration reduced the plasma level of blood urea-nitrogen, creatinine and uric acid, compared to acetaminophen-exposed animals (group U). In renal disease, the serum urea accumulates because the rate of serum urea production exceeds the rate of clearance. Elevation of urea and creatinine levels in the serum is taken as the index of nephrotoxicity. Creatinine, on the other hand, is mostly derived from endogenous sources by tissue creatinine breakdown (Abdel-Zaher et al. 2007). Thus serum urea concentration is often considered a more reliable renal function predictor than plasma creatinine. In the present study, administration of hepatotoxic and nephrotoxic doses of acetaminophen to rats resulted in development of oxidative stress damaging hepatic and renal tissues. Acetaminophen induced nephrotoxicity showed a significant ($p < 0.05$) increase in the plasma urea and creatinine concentrations in the group U (acetaminophen induced) rats when compared to the normal group (group NC). Moreover, oral administration of *L. ingluviei* ADK10 significantly ($p < 0.05$) decreased plasma urea and creatinine in the group UP when compared to the group U (Fig. 3b). This study clearly indicated that suitable bacteriotherapy can be efficient for lowering the uremic toxins in acetaminophen induced uremic rats. This is low cost and cheap. In future this probiotic lactobacillus can be used as a therapeutic food supplement for kidney failure, other kidney disease and kidney transplanted patients.

Analysis of faecal enteric bacteria

Significant decrease occurred in the counts of enteric bacteria in the stool of group fed with *L. ingluviei* ADK10 (Table 4) after 14 days of daily feeding with 1×10^9 CFU bacteria for 14 days, as compared with the group NC. This may have been due to the daily intake of *L. ingluviei* ADK10 which have antimicrobial activity *in vitro*. This result may be due to *L. ingluviei* ADK10 adhering to the intestinal membrane or the intestinal environment serving to create worse survival conditions for enteric bacteria.

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